INDUCED RESPONSES AND DEVELOPMENTAL CHANGES OF DIRECT AND INDIRECT DEFENSES IN THE YOUNG LEAVES OF THE NEOTROPICAL TREE GENUS *INGA*

by

Ryan James Bixenmann

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STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Ryan James Bixenmann**

has been approved by the following supervisory committee members:

Phyllis D. Coley	, Chair	**4/27/2011** Date Approved
Thomas A. Kursar	, Member	**4/27/2011** Date Approved
M. Denise Dearing	, Member	**4/27/2011** Date Approved
Donald H. Feener Jr.	, Member	**4/27/2011** Date Approved
Colleen G. Farmer	, Member	**4/27/2011** Date Approved

and by **Neil Vickers**, Chair of the Department of **Biology**

and by Charles A. Wight, Dean of The Graduate School.

ABSTRACT

Plant-herbivore interactions are the most common macroscopic interactions on the planet and are an important mechanism for maintaining species coexistence. Tropical young leaves are under higher and more constant herbivore pressure than their temperate zone counterparts. Therefore tropical leaves are predicted to invest in constitutive defenses (always expressed) over induced defenses (expressed only when herbivores are present). However, there is little empirical evidence to support this hypothesis. In addition, little is known about developmental changes in defense investment during tropical young leaf development regardless of herbivore presence.

In this dissertation, I demonstrated that younger leaves are better defended than more mature leaves in four species of the Neotropical tree genus *Inga*. In addition to chemical defenses, *Inga* species produce nectar on their leaf surfaces to attract ants that in turn patrol the leaves and remove herbivores. Through natural observations and experimental nectar manipulation I demonstrated that plants with higher nectar production rates received more ant bodyguards. I then showed that investment in both ant defense and chemical defense were highest in the youngest leaves. While investment in both of these defenses continued during young leaf development, the relative investment per leaf tissue decreased significantly as leaves expanded.

Through experimental manipulation of ant presence and herbivore presence I also demonstrated that tropical young leaves have a limited capacity to induce their defenses. I

showed that plants can increase their nectar production but not their defense chemistry when ants are present. However, I showed that there was no change in nectar production and a very limited change in chemical defenses when herbivores were present. This evidence supports the hypothesis that tropical young leaves are primarily protected by constitutive defenses.

This is the first report of tropical plants investing in constitutive rather than induced defenses, and supports predictions of the induced resistance hypothesis that are rarely tested. Furthermore, divergent defenses among *Inga* species appear to be species level traits and not plastic responses. Thus, my results support the hypothesis that a diversity of antiherbivore defenses among plant species is an important mechanism in maintaining species coexistence.

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CHAPTER 1

INTRODUCTION

Plant-herbivore interactions are the most common macroscopic interactions on the planet and are important in structuring communities and maintaining species coexistence (Schoonhoven et al. 2005, Becerra 2007, Kursar et al. 2009, Utsumi and Ohgushi 2009, Utsumi 2011). Not surprisingly, herbivores prefer plant tissue that is high in nitrogen, soft, and easily digested. However, the loss of these important nutrients and stored assimilated carbon comes at a fitness cost to plants. For example, herbivory is a positively correlated with tree mortality and negatively correlated with plant growth and seed production (Marquis 1984, Eichhorn et al. 2010). Therefore plants have developed several defense strategies.

Plants invest in defenses to reduce tissue loss to herbivores, but ultimately the most effective defense is leaf toughness (Coley 1983, Lowman 1983). Toughness lowers the nutritional quality of leaf tissue by lowering the ratio of nutrients to fibrous, indigestible carbohydrates (Kursar and Coley 2003). In order to expand, young leaves are inherently soft and high in nitrogen. Consequently, they suffer the most damage (Coley 1983, Coley and Aide 1991, Kursar and Coley 2003, Brunt et al. 2006). Phenological defenses minimize the exposure time of young leaves to herbivores by either growing quickly or growing when herbivores are less abundant (Kursar and Coley 2003). However, when leaves are young and the most susceptible to damage, plants invest in

direct and indirect defenses to reduce herbivore damage. Direct defenses, such as toxic secondary metabolites and physical barriers to herbivores (e.g., spines, hairs, trichomes), are produced by plants and directly affect herbivore performance. Indirect defenses are signals produced by the plant that attract predators of herbivores (Kost and Heil 2008). Because these defenses rely on other biotic agents they are often referred to as biotic defenses (Ness 2003).

Facultative ant-plant mutualisms are one of the most common biotic defenses. In all ant-plant mutualisms, food rewards attract ants to patrol leaves and act as bodyguards (Bentley 1976, Bentley 1977). In obligate ant-plant mutualisms, plants provide specialized housing structures, specialized food rewards, and typically house one ant colony for a long period of time. In contrast, facultative ant-plants typically offer a generalized food reward (usually extrafloral nectar) and no specialized nesting structures. Consequently, facultative ant-plants have high turnover rates of ant species that are on the order of hours to days (Heil and McKey 2003). Roughly a third of tree species in tropical rain forests facultatively attract ant bodyguards whereas less than 10% of the trees have obligate ant partners (Coley and Aide 1991, Schupp and Feener 1991). Despite this, facultative ant-plants are less studied and consequently the host-plant dynamics are poorly understood.

Because all defenses require a resource investment, plants are faced with the dilemma of investing in growth or defense (Herms and Mattson 1992). High investment in defense may result in reduced loss to herbivores but a low investment in reproduction and thus lower fitness. In contrast, high investment in growth may result in more assimilated carbon for reproduction, however, without protection the stored carbon can

be easily lost to herbivores. The outcomes of these two investment strategies are context dependent and addressed by several hypotheses: Optimal Defense, Resource Availability, Carbon/Nutrient, and Induced Resistance (Rhoades 1979, Bryant et al. 1983, Coley et al. 1985, Karban and Baldwin 1997). These hypotheses predict that plants balance their investments in growth and defense based on available resources, herbivore pressure, tissue value, and cost of defense. They all predict that plants will invest in the strategies that provide the highest return on their investment (Stamp 2003).

Induced resistance is a cost-saving strategy in which defenses are only expressed in the presence of herbivores. Consequently, induced defenses are predicted to evolve when herbivory is variable (Karban and Baldwin 1997, Karban et al. 1999). In contrast, constitutive defenses are continuously expressed regardless of herbivore presence (Karban and Baldwin 1997). Induced defenses are therefore more cost efficient because limited resources are only invested in defenses when they are needed. However, delays in the induction of a defense could result in herbivore damage and therefore constitutive defenses would be more adaptive if herbivory is invariable. Although many advancements have been made in our understanding of induced defenses, they are almost entirely from temperate or agricultural plants (Karban et al. 1997, Boege 2004).

Tropical plants experience greater herbivore pressure and invest more in defenses than temperate plants (Coley and Aide 1991, Coley and Barone 1996). In particular, tropical young leaves, which are vulnerable for only a few weeks, are under the highest herbivore pressure and receive 70% of a leaf's lifetime herbivore damage. Under such high, constant herbivore pressure, even a delay of one day to induce defenses could result in substantial damage. Furthermore, the probability of attack among young leaf flushes is

high and therefore would make constitutive defenses more adaptive. However, there are limited reports from tropical plants and many of those are from seasonal tropical forests where herbivore pressure is seasonally sporadic (Boege 2004, Massey et al. 2005).

I have chosen to examine how these hypotheses of plant defense apply in a diverse Neotropical genus, *Inga* (Fabaceae), growing in Panama. *Inga* has over 300 species and ranges from southern Mexico to Southern Brazil (Pennington 1997). The genus is one of the most species-rich and abundant tree genera in local communities (Kursar et al. 2009). The defense chemistry for the common species of *Inga* on Barro Colorado Island, Panama, is diverse and includes chemical, phenological, and biotic defenses (Koptur 1984, 1985, Coley et al. 2005, Kersch and Fonseca 2005, Lokvam and Kursar 2005, Brenes-Arguedas et al. 2006, Lokvam et al. 2006, Brenes-Arguedas et al. 2008). Within the genus, there are species that have regular leaf expansion and species that have rapid leaf expansion where leaves spend very little time in the vulnerable young leaf state. Both fast- and slow-expanding species invest in defense chemistry and extrafloral nectar production. Chemical defenses among the closely related species within a community are more diverse than is expected by chance (Kursar et al. 2009). However it is not clear if this is due to selection by the local herbivore community or to plastic responses such as induced defenses. In addition, there is variation in ant visitation rates among *Inga* species (Brenes-Arguedas et al. 2008). The recent divergence and high diversity of defensive traits among closely related species of *Inga* species in a single tropical forest make it an ideal system for investigating the effects of environmental factors on defense expression.

In this thesis I address the following questions:

1) Does variation in extrafloral nectar production affect ant visitation on the young leaves of *Inga* species?
2) Do plants in the genus *Inga* invest in induced defenses to protect young leaves?
3) How does investment among direct and indirect defenses change during young leaf development?

In Chapter 2, I demonstrated the positive relationship between extrafloral nectar production by *Inga* species and ant bodyguards. In addition, I demonstrated the plasticity of extrafloral nectary production to biotic and abiotic factors. I established that nectar production is a species-level trait and that variation in nectar production among *Inga* species positively correlates with ant visitation. In addition, using natural nectar production and artificial nectar rewards, I showed the positive relationship between investment in nectar production and ant visitation. I then demonstrated that nectar production can be induced by ant presence and when plants are grown in gaps. Because these are facultative partners and an individual plant's ant defense is dependent on the local ant community, it is beneficial for the plants to respond to ant presence by increasing nectar production to attract more defenders. However, in the absence of ants, investing in nectar production may not only be a poor investment, but may also attract and feed herbivores. Thus, investment in indirect defenses can be regulated by the plant and investments in these defenses attract more defenders.

Chapter 3 showed that the direct chemical defenses in four *Inga* species were canalized species-level traits that are constitutively expressed. I examined three defensive secondary metabolites (phenolics, saponins, and tyrosine) and demonstrated that only

fast-expanding species could induce their chemical defenses in response to herbivore presence. However, with the exception of one unique chemical defense (tyrosine), the induced amount was limited to phenolics and was a small increase (20-25%). While other Fabaceae species can induce saponins (Agrell et al. 2003), they were not induced in *Inga*. Within the genus, the capacity to induce was negatively correlated with the probability of attack and with investment in constitutive defenses. Because the capacity to induce in the genus *Inga* was small compared to examples from temperate plants, it suggests a decreased investment in induced defenses in aseasonal tropical forests.

In addition to the phenotypic responses of defensive chemistry, I examined the response of jasmonic acid (JA), an important damage signaling hormone, in response to herbivore damage. Jasmonic acid is upregulated when tissue is damaged and elicits upregulation of defenses (Koo and Howe 2009). My results demonstrated that JA did not significantly increase in plants that had been damaged. However, all four *Inga* species had a positive trend of increased JA when they were damaged. I explored potential reasons for the lack of a significant result and suggested that my experimental design was not appropriate to effectively quantify JA accumulation. Alternatively, the positive trend between JA and herbivore presence and the limited capacity to induce within the genus *Inga* suggests that *Inga* species sense damage through the JA pathway, but selection may have acted downstream of the JA pathway to block an induced response in young leaves.

While young leaves did not change their chemistry or nectar production in response to herbivore presence, there was a large change in defense chemistry and nectar production during natural leaf development. In Chapter 4 I showed that there was continued investment in defenses during young leaf expansion but that the investment per

leaf tissue (mass or area) decreased as leaves expanded. Tyrosine was the only exception to this relationship. However, I speculated that tyrosine can be reallocated unlike the other defenses and therefore was regulated differently. Taken together, the large investment per leaf tissue in the youngest leaves, the high nitrogen content in the youngest leaves, and the large impact of tissue loss on the youngest leaves suggest that younger, smaller leaves have the highest potential value and are therefore protected more thoroughly. Furthermore, the lack of any trade-offs among the defenses suggests that young leaf tissue is the most valuable leaf stage and that multiple defenses may have an additive effect rather than a redundant effect when protecting valuable tissue against a diversity of herbivores.

The ability of facultative ant-plants to respond to ant presence suggests that host-plants are dynamic and can actively invest in ant defense based on the local ant community. In addition, this is the first report to examine induced direct and indirect defenses in a tropical forest and is one of only a few studies to report on trade-offs among defenses during young leaf expansion. Taken together these results indicate the importance of young leaf tissue. Furthermore, the results suggest that herbivory is sufficiently high and constant to make constitutive defenses more adaptive despite the costs associated with continued expression of a defense.

References

Agrell, J., W. Oleszek, A. Stochmal, M. Olsen, and P. Anderson. 2003. Herbivore-induced responses in alfalfa (*Medicago sativa*). Journal of Chemical Ecology **29**:303-320.

Becerra, J. X. 2007. The impact of herbivore-plant coevolution on plant community structure. Proceedings of the National Academy of Sciences **104**:7483-7488.

Bentley, B. L. 1976. Plants bearing extrafloral nectaries and the associated ant community: Interhabitat differences in the reduction of herbivore damage. Ecology **57**:815-820.

Bentley, B. L. 1977. Extrafloral nectaries and protection by pugnacious bodyguards. Annual Review of Ecology and Systematics **8**:407-427.

Boege, K. 2004. Induced responses in three tropical dry forest plant species - direct and indirect effects on herbivory. Oikos **107**:541-548.

Brenes-Arguedas, T., P. D. Coley, and T. A. Kursar. 2008. Divergence and diversity in the defensive ecology of *Inga* at two Neotropical sites. Journal of Ecology **96**:127-135.

Brenes-Arguedas, T., M. W. Horton, P. D. Coley, J. Lokvam, R. A. Waddell, B. E. Meizoso-O'Meara, and T. A. Kursar. 2006. Contrasting mechanisms of secondary metabolite accumulation during leaf development in two tropical tree species with different leaf expansion strategies. Oecologia **149**:91-100.

Brunt, C., J. Read, and G. D. Sanson. 2006. Changes in resource concentration and defence during leaf development in a tough-leaved (*Nothofagus moorei*) and soft-leaved (*Toona ciliata*) species. Oecologia **148**:583-592.

Bryant, J. P., F. S. Chapin, and D. R. Klein. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos **40**:357-368.

Coley, P. D. 1983. Herbivory and defensive characteristics of tree species in a lowland tropical forest. Ecological Monographs **53**:209-234.

Coley, P. D. and T. M. Aide. 1991. Comparisons of herbivory and plant defenses in temperate and tropical broad-leaved forests. Pages 25-49 *in* P. W. Price, T. M. Lewinsohn, G. W. Fernandes, and W. W. Benson, editors. Plant-animal interactions: Evolutionary ecology in tropical and temperate regions. John Wiley & Sons, Inc., New York.

Coley, P. D. and J. A. Barone. 1996. Herbivory and plant defenses in tropical forests. Annual Review of Ecology and Systematics **27**:305-335.

Coley, P. D., J. P. Bryant, and F. S. Chapin. 1985. Resource availability and plant antiherbivore defense. Science **230**:895-899.

Coley, P. D., J. Lokvam, K. Rudolph, K. Bromberg, T. E. Sackett, L. Wright, T. Brenes-Arguedas, D. Dvorett, S. Ring, A. Clark, C. Baptiste, R. T. Pennington, and T. A. Kursar. 2005. Divergent defensive strategies of young leaves in two species of *Inga*. Ecology **86**:2633-2643.

Eichhorn, M. P., R. Nilus, S. G. Compton, S. E. Hartley, and D. F. R. P. Burslem. 2010. Herbivory of tropical rain forest tree seedlings correlates with future mortality. Ecology **91**:1092-1101.

Heil, M. and D. McKey. 2003. Protective ant-plant interactions as model systems in ecological and evolutionary research. Annual Review of Ecology Evolution and Systematics **34**:425-453.

Herms, D. A. and W. J. Mattson. 1992. The dilemma of plants: To grow or defend. Quarterly Review of Biology **67**:283-335.

Karban, R., A. Agrawal, and M. Mangel. 1997. The benefits of induced defenses against herbivores. Ecology **78**:1351-1355.

Karban, R., A. A. Agrawal, J. S. Thaler, and L. S. Adler. 1999. Induced plant responses and information content about risk of herbivory. Trends in Ecology & Evolution **14**:443-447.

Karban, R. and I. T. Baldwin. 1997. Induced responses to herbivory. University of Chicago Press, Chicago.

Kersch, M. F. and C. R. Fonseca. 2005. Abiotic factors and the conditional outcome of an ant-plant mutualism. Ecology **86**:2117-2126.

Koo, A. J. K. and G. A. Howe. 2009. The wound hormone jasmonate. Phytochemistry **70**:1571-1580.

Koptur, S. 1984. Experimental evidence for defense of *Inga* (Mimosoideae) saplings by ants. Ecology **65**:1787-1793.

Koptur, S. 1985. Alternative defenses against herbivores in *Inga* (Fabaceae: Mimosoideae) over an elevational gradient. Ecology **66**:1639-1650.

Kost, C. and M. Heil. 2008. The defensive role of volatile emission and extrafloral nectar secretion fro lima bean in nature. Journal of Chemical Ecology **34**:2-13.

Kursar, T. A. and P. D. Coley. 2003. Convergence in defense syndromes of young leaves in tropical rainforests. Biochemical Systematics and Ecology **31**:929-949.

Kursar, T. A., K. G. Dexter, J. Lokvam, R. T. Pennington, J. E. Richardson, M. G. Weber, E. T. Murakami, C. Drake, R. McGregor, and P. D. Coley. 2009. The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus *Inga*. Proceedings of the National Academy of Sciences **106**:18073-18078.

Lokvam, J., T. Brenes-Arguedas, J. S. Lee, P. D. Coley, and T. A. Kursar. 2006. Allelochemic function for a primary metabolite: the case of L-tyrosine hyper-production in *Inga umbellifera* (Fabaceae). American Journal of Botany **93**:1109-1115.

Lokvam, J. and T. A. Kursar. 2005. Divergence in structure and activity of phenolic defenses in young leaves of two co-occurring *Inga* species. Journal of Chemical Ecology **31**:2563-2580.

Lowman, M. D. 1983. Variation in leaf toughness and phenolic content among five species of Australian rain forest trees. Australian Journal of Ecology **8**:17-25.

Marquis, R. J. 1984. Leaf herbivores decrease fitness of a tropical plant. Science **226**:537-539.

Massey, F. P., M. C. Press, and S. E. Hartley. 2005. Long- and short-term induction of defences in seedlings of *Shores leprosula* (Dipterocarpaceae): Support for the carbon:nutrient balance hypothesis. Journal of Tropical Ecology **21**:195-201.

Ness, J. H. 2003. *Catalpa bignonioides* alters extrafloral nectar production after herbivory and attracts ant bodyguards. Oecologia **134**:210-218.

Pennington, T. D. 1997. The Genus Inga. Royal Botanic Gardens, Kew.

Rhoades, D. F. 1979. Evolution of plant chemical defense against herbivores. . Pages 1-55 *in* G. A. Rosenthal and D. H. Janzen, editors. Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, New York:.

Schoonhoven, L. M., J. J. A. Van Loon, and M. Dicke. 2005. Insect-plant biology. Oxford University Press, USA.

Schupp, E. W. and D. H. Feener. 1991. Phylogeny, lifeform, and habitat dependence of ant-defended plants in a Panamanian forest.*in* C. R. Huxley and D. F. Cutler, editors. Ant-plant interactions. Oxford University Press, USA.

Stamp, N. 2003. Out of the quagmire of plant defense hypotheses. Quarterly Review of Biology **78**:23-55.

Utsumi, S. 2011. Eco-evolutionary dynamics in herbivorous insect communities mediated by induced plant responses. Population Ecology **53**:23-34.

Utsumi, S. and T. Ohgushi. 2009. Community-wide impacts of herbivore-induced plant regrowth on arthropods in a multi-willow species system. Oikos **118**:1805-1815.

CHAPTER 2

IS EXTRAFLORAL NECTAR PRODUCTION INDUCED BY HERBIVORES OR ANTS IN A TROPICAL FACULTATIVE ANT-PLANT MUTUALISM?

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Is extrafloral nectar production induced by herbivores or ants in a tropical facultative ant-plant mutualism? Oecologia 165:417-425 (2011)

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PLANT-ANIMAL INTERACTIONS - ORIGINAL PAPER

Is extrafloral nectar production induced by herbivores or ants in a tropical facultative ant–plant mutualism?

R. J. Bixenmann · P. D. Coley · T. A. Kursar

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Abstract Many plants use induced defenses to reduce the costs of antiherbivore defense. These plants invest energy in growth when herbivores are absent but shunt energy to defense when herbivores are present. In contrast, constitutive defenses are expressed continuously regardless of herbivore presence. Induction has been widely documented in temperate plants but has not been reported from tropical plants. Most tropical plants have higher, more constant herbivore pressure than temperate plants. In this situation, it is hypothesized that constitutive defenses rather than induced defense would be favored. Using natural herbivores of four species of *Inga* saplings on Barro Colorado Island, Panama, herbivore presence was crossed with ant presence to determine their effects on extrafloral nectar production. Analysis of nectar samples revealed that *Inga* species do not induce nectar production in response to herbivores. This result is not due to an inability of the plants to respond, as the plants in this study increased nectar production in response to light and ant presence. Contrary to most induction experiments with temperate ecosystem plants, these results demonstrate that tropical plants do not induce one type of defense, and they suggest that the most adaptive defense strategies are different for the two ecosystems.

Keywords Constitutive defense · *Inga* · Facultative mutualism · Plant–insect interaction · Herbivory

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R. J. Bixenmann (✉) · P. D. Coley · T. A. Kursar
Department of Biology, University of Utah,
Salt Lake City, UT 84112-0840, USA
e-mail: r.bixenmann@utah.edu

Introduction

Plants have evolved a battery of antiherbivore defenses in response to the selective pressure exerted by herbivore damage. Defenses such as toxic chemicals, physical structures, and rewards that attract enemies of herbivores are believed to be costly for plants to produce, and the resources used for defense usurp resources from other plant activities such as growth and reproduction. Thus, plants are faced with the classic dilemma of investing in growth or in defense (Herms and Mattson 1992). The optimal defense hypothesis addresses this dilemma by predicting that plants will invest in defenses only when they are cost-effective and will reduce investment in redundant defenses (Stamp 2003).

Induced defenses have received considerable attention in the past decade as a cost-efficient defense strategy. This defense strategy permits plants to maintain a low level of defense that can be upregulated in response to environmental cues (biotic or abiotic) in order to reduce future herbivore damage. Induced defenses are predicted to evolve only when reducing the defense would enhance fitness in the absence of herbivores and when the risk of herbivore damage is variable and a reliable cue exists to trigger the induced response (Karban et al. 1999; Rohde and Wahl 2008). In contrast, constitutive defenses (defenses that are continuously expressed regardless of herbivore presence) are predicted to be advantageous over induced defenses when herbivore pressure is constantly high.

Most examples of defenses induced by herbivores have been in plants from temperate ecosystems (Boege 2004). Temperate environments experience seasonal herbivore loads that may predict future herbivore pressure and act as a reliable cue for plants. There is, however, a lack of evidence demonstrating herbivore-induced defenses from

tropical forests. Tropical plants, especially young leaves, experience higher percent herbivore damage than temperate plants (Coley and Aide 1991). Young leaves of tropical plants are the most susceptible to herbivore consumption because they are softer and have higher relative nutrient and water contents than mature leaves (Coley and Kursar 1996). Roughly 70% of a tropical leaf's lifetime damage occurs in this short period of intense growth (Coley and Barone 1996). Thus, young leaves in tropical forests are under high, almost constant herbivore pressure, which should favor constitutive defenses over induced defenses.

Facultative ant–plant mutualisms are common antiherbivore defenses in tropical forests (Schupp and Feener 1991). In return for extrafloral nectar, ants serve as bodyguards and patrol young leaf surfaces, removing or deterring unwanted pests. In contrast to obligate ant–plant mutualisms, facultative mutualisms provide food but not housing for ants, which can result in rapid changes in the abundance and identity of the ant partners (Heil and McKey 2003). Consequently, we predict that extrafloral nectar in facultative ant–plants should respond to ant presence by changing the nectar production rate.

In this paper we examine the effects of experimentally manipulated herbivore and ant presence on the production of extrafloral nectar in four Neotropical tree species in the genus *Inga* (Fabaceae) on Barro Colorado Island, Panama. At the genus level, *Inga* has a diverse array of chemical and phenological defenses (Brenes-Arguedas et al. 2006; Coley et al. 2005; Lokvam et al. 2006; Lokvam et al. 2004; Lokvam and Kursar 2005). Chemical defenses are toxic to herbivores and phenological defenses, such as rapid leaf expansion, allow the leaves to spend less time in the vulnerable young leaf state (Coley et al. 2005). Most species of *Inga* also have extrafloral nectaries that produce nectar and use ant protection to varying degrees (Brenes-Arguedas et al. 2006; Koptur 1984; Koptur 1985). Extrafloral nectaries in *Inga* are located on the rachis between leaflet pairs and only secrete nectar when the leaves are young and expanding. It has been shown that extrafloral nectary traits are heritable and that the quantity and quality of nectar can influence the patrolling behavior of ants (Apple and Feener 2001; Bluthgen et al. 2004; Bronstein et al. 2006; Koptur 1992; Koptur 1994; Koptur 2005; Rudgers 2004; Rudgers and Gardener 2004). We therefore conducted a survey of extrafloral nectar production in *Inga* and how changes in this production rate influenced ant patrolling behavior. There is, however, a possible feedback loop in some systems between ant presence and nectar production. Heil et al. (2000) have shown that ant presence itself induced the production of extrafloral nectar. Therefore, we manipulated herbivore presence and ant presence to determine if either induces extrafloral nectar production on the young leaves of *Inga*.

Methods

Field research was conducted on Barro Colorado Island (BCI), Panama from May 2007 to November 2007, and again in the same months in 2008. BCI is located in the Panama Canal (9°N80°W) and is maintained by the Smithsonian Tropical Research Institute. The island is a tropical moist lowland forest that experiences a 4-month dry season (January to April) (Croat 1978; Holdridge et al. 1971; Leigh 1999). For *Inga*, most young leaves are produced during the rainy season (Coley and Kursar, pers. observ.). This is also the season that herbivores and their predators (ants) are more abundant in both gaps and understories (Richards and Windsor 2007).

Ant visitation and nectar quality and quantity

Nectar surveys were conducted from May to August of 2007 and 2008. Six *Inga* species along the trails on BCI were selected: *I. acuminata* ($n = 3$), *I. marginata* ($n = 44$), *I. multijuga* ($n = 23$), *I. pezizifera* ($n = 22$), *I. umbellifera* ($n = 40$), and *I. vera* ($n = 5$). One branch per individual tree between 1 and 4 m tall was sampled at one time. Most branches had only one young leaf. When multiple young leaves per branch were sampled, the average nectar production of those leaves was used as a response for the individual tree. To collect nectar from the young leaves, nectaries were washed with distilled water to remove accumulated nectar. Then the entire leaf was placed in a plastic bag to prevent rain or insects from removing nectar. We did not use mesh bags because they would not have prevented disturbance by rain. While plastic bags may influence temperature and humidity, the bags were not airtight and samples were collected mostly in the shaded understory and during the rainy season when air temperatures were cooler. In addition, condensation on the inside of the bags was rarely observed in either habitat (gap or understory), indicating no significant heat difference between the inside and outside of the bag. After 24 h, the nectar was collected and its volume measured using microcapillary tubes (initial collection). To collect any residual nectar, one microliter drops of distilled water were placed on nectaries, collected, and added to the initial collection. The nectar was collected into glass GC vials, dried under vacuum, and frozen at −50°C until analysis.

An HP 6890 gas chromatograph with a DB-1 capillary column and FID was used to identify and quantify the sugars in the nectar. The majority of the components in the nectar were the sugars fructose, glucose, and sucrose. The dried nectar samples were dissolved in 50 μL of pyridine. Due to the polarity of the sugars, the samples were derivatized by adding 50 μL of BSTFA with 1% TCMS to the pyridine solution and allowed to sit for 10 h. External

standards and calibration curves were used to identify and quantify the three sugars in the nectar. The total mass of the three sugars was summed for each sample and corrected by the number of nectaries per leaf over a 24 h period (μg sugar nectary^{-1} 24 h^{-1}).

Based on the survey of natural nectar production, artificial nectar solutions were prepared and placed on adult leaves of four *Inga* species: *I. marginata*, *I. multijuga*, *I. pezizifera*, and *I. umbellifera*. Adult leaves were used as they no longer produce nectar naturally. Solutions maintained a sugar ratio of one [i.e., 1 mg sucrose/(0.5 mg fructose + 0.5 mg glucose)]. Five separate concentrations were prepared: 0 μg μL^{-1} ($n = 30$); 2 μg μL^{-1} ($n = 29$); 20 μg μL^{-1} ($n = 27$); 180 μg μL^{-1} ($n = 28$); and 950 μg μL^{-1} ($n = 28$). A concentration was randomly chosen for each plant included. A plant was only used once and only one leaf on the plant was observed. One microliter of the selected solution was placed on the two proximal nectaries on the rachis. After an hour, the ants on leaf surfaces and at nectaries were counted.

Induction

To assess if herbivores could induce defenses in young leaves, we randomly assigned plants along the trails of BCI to either an herbivore damage treatment (plus-caterpillar) or an herbivore-free treatment in which herbivores were removed and they received no herbivore damage (minus-caterpillar). Previous research has shown that mechanical wounding alone does not mimic herbivore damage (Arimura et al. 2005); therefore, natural herbivores were used to elicit an induction response in young leaves of four species of *Inga*: *I. marginata* ($n = 33$), *I. pezizifera* ($n = 20$), *I. multijuga* ($n = 25$), and *I. umbellifera* ($n = 27$). The most common herbivore on *Inga* species on BCI is a black-headed leaf roller (Gelechiidae, species not identified) (Kursar et al. 2006). Second- and third-instar gelechiids were collected from non-focal *Inga* plants and moved to plus-herbivore plants.

To test if ants affect expression of defenses by plants, individual plants in each of the two herbivore treatments were assigned to either a treatment with normal ant visitation to leaves (plus-ants) or to a treatment where ant access to leaves was restricted by the addition of a sticky barrier (Tanglefoot) on the branch (minus-ants). In addition, plants were sampled in gaps and in the understory, and measures were taken to ensure that each treatment was evenly crossed with these two light categories for each species. These three factors—herbivore treatment, light and ant treatment—were fully crossed, and an individual plant only experienced one level of each treatment.

All of the treatments were applied to young leaves. Plants were incorporated into the experiment before their young leaves reached 15% of their average adult leaf area. If there was any pre-existing damage, plants were not included and plants were not reused. Treatments were maintained until the leaves reached 80% of their adult size (the size when the majority of herbivory has occurred and defense chemicals are high; Kursar and Coley 2003) or over half of their leaf tissue was damaged. At these terminal time points, nectar was collected for laboratory analysis (see above for the collection method).

Individual plants were treated as units of replication. When a plant had nectar collected from multiple leaves, the values for the multiple collections were averaged and used for an individual plant. Only values from leaves that were in the targeted size range (60–80% of adult size) were used. An analysis of variance was run with all three factors (herbivore presence, ant presence, and light) using plant species as a blocking variable and an alpha level of 0.05 (Table 1).

Results

Ant visitation and nectar quality and quantity

The predominant components of extrafloral nectar of *Inga* species were sucrose, fructose and glucose. The ratio of these three sugars is typically expressed as the sucrose-to-hexose ratio [mg sucrose/(mg fructose + mg glucose)]

Table 1 ANOVA summary table for the effects of induced responses by herbivores, ants, light, and their interactions

Source	DF	Mean square	*F* value
Ant presence	1	437,839	4.2679*
Caterpillar presence	1	17,352	0.1691
Light	1	1,096,620	10.6894**
Inga species (block)	3	331,142	3.2278*
Ant × caterpillar	1	19,411	0.1892
Ant × light	1	67,271	0.6557
Caterpillar × light	1	176,381	1.7193
Ant × *Inga* spp.	3	48,476	0.4725
Caterpillar × *Inga* spp.	3	101,631	0.9907
Light × *Inga* spp.	3	592,899	5.7793**
Ant × caterpillar × light	1	72,688	0.7085
Ant × caterpillar × *Inga* spp.	3	29,792	0.2904
Ant × light × *Inga* spp.	3	55,736	0.5433
Caterpillar × light × *Inga* spp.	2	13,581	0.1324
Ant × caterpillar × light × *Inga* spp.	2	401	0.0039
Residuals	75	102,589	

Plant species was used as a blocking variable to remove variation due to different natural history traits

* $P < 0.05$, ** $P < 0.01$

(Baker and Baker 1982; Baker and Baker 1983). The average sugar ratio for the seven study species was 0.48 (when outliers were removed), with no significant difference among species. We were unable to positively identify and quantify any additional components of the nectar, such as amino acids.

There was no significant correlation between the volume of nectar produced and the concentration of the nectar. There was, however, a significant positive relationship between the volume of nectar produced and the total mass of sugar produced (slope = 0.85, $r^2 = 0.72$, $P < 0.001$). Thus, we chose to use total mass of sugar per nectary per 24 h period (μg nectary^{-1} 24 h^{-1}) rather than volume for all further comparisons, as it was less prone to environmental fluctuation and is the component of the nectar that is most attractive to ants (Gonzalez-Teuber and Heil 2009). On average, the amount of sugar produced was 168 μg nectary^{-1} 24 h^{-1}. However, this varied among plant species. *Inga acuminata* produced no nectar (0.0 μg nectary^{-1} 24 h^{-1}) while *Inga marginata* produced the most nectar (277 μg nectary^{-1} 24 h^{-1}; Fig. 1).

Increases in natural and artificial nectar rewards significantly increased ant visitation to leaf surfaces. Natural nectar production for seven species of *Inga* was positively correlated with natural ant visitation ($r^2 = 0.62$, $P = 0.035$, Fig. 2). Furthermore, when artificial nectar was placed in the inactive extrafloral nectaries of mature leaves, the number of ants increased as the amount of sugar increased ($P < 0.001$, Fig. 3). In addition, there was a significant interaction between concentration and observed ant location ($P = 0.0045$): at low concentrations, an equal number of ants visited nectaries as visited leaf surfaces, but at high concentrations, more ants visited nectaries than visited leaf surfaces (Fig. 3).

Induction

We examined the extent to which nectar traits were induced by herbivore presence, ant visitation, and light availability. For this experiment we focused on four species: *I. marginata*, *I. multijuga*, *I. pezizifera*, and *I. umbellifera*.

As in the nectar survey, concentration did not change significantly among the treatments; nor was it correlated with the volume of nectar produced. Sugar ratios showed no significant differences among the treatments or species. Overall, the average was 0.46, indicating there were similar amounts of sucrose to the two monomers, fructose and glucose. However, total mass of sugar was positively correlated with volume (slope = 0.74, $r^2 = 0.55$, $P < 0.01$). Thus, mass per nectary per 24 h period was used for the statistical analysis.

On average across all treatments, *I. multijuga* produced the most nectar in a 24 h period (358 μg nectary^{-1} 24 h^{-1}), followed by *I. pezizifera* (167 μg nectary^{-1} 24 h^{-1}) and *I. marginata* (147 μg nectary^{-1} 24 h^{-1}), with *I. umbellifera* producing the smallest amount (103 μg nectary^{-1} 24 h^{-1}). Therefore, plant species was used as a blocking variable for analysis of variance. Despite a large amount of damage (21% on plus-herbivore plants), herbivore presence did not induce an increase in nectar production (Fig. 4a). However, ant presence and high light did induce an increase in nectar production ($P = 0.042$ and 0.0016, respectively) (Fig. 4b, c).

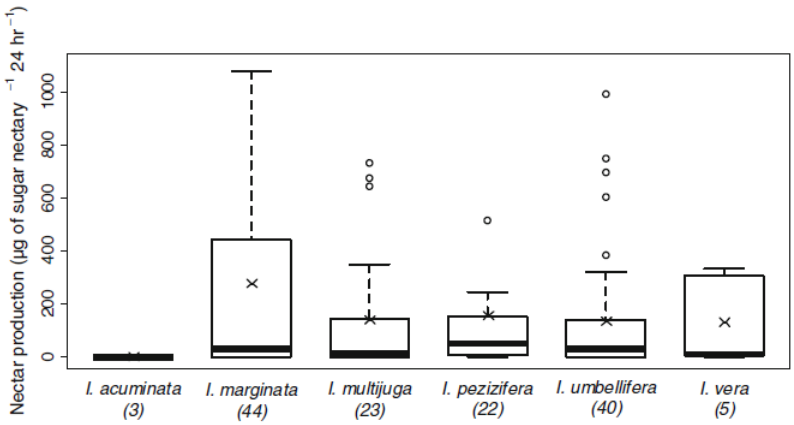

Fig. 1 The natural variation in nectar production among six *Inga* species: *I. acuminata* ($n = 3$), *I. marginata* ($n = 44$), *I. multijuga* ($n = 23$), *I. pezizifera* ($n = 22$), *I. umbellifera* ($n = 40$), and *I. vera* ($n = 5$). The *black bars* in the middle of each *box* represent the median value for the species, the *ends of the box* represent the upper and lower quartiles, and the *whiskers* represent the upper and lower non-outlier values. The *crosses* represent the mean value for each species. Sample sizes are in *parentheses* below each species name

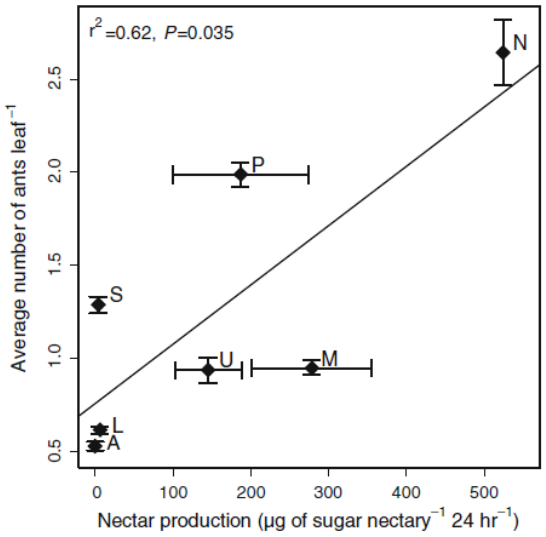

Fig. 2 The average number of protective ants on leaf surfaces in relation to a species' investment in extrafloral nectar production ($r^2 = 0.62$, $P = 0.035$). *Points* in the graph represent the average number of naturally occurring ants observed during spot counts and the average mass of sugar produced per nectary in a 24 h period for seven species: *I. acuminata* (*A*), *I. laurina* (*L*), *I. sapindoides* (*S*), *I. umbellifera* (*U*), *I. pezizifera* (*P*), *I. marginata* (*M*), *I. nobilis* (*N*). *Letters* next to each point represent the species code, and the *error bars* are standard errors of the mean for both axes

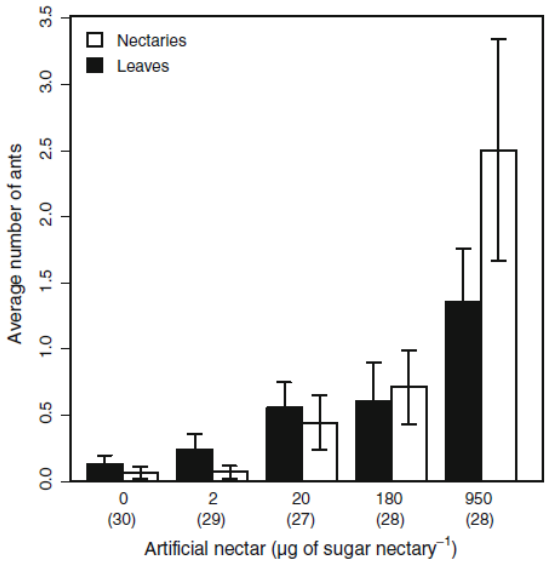

Fig. 3 The average number of ants on leaf surfaces and at nectaries in relation to artificial nectar concentration. One microliter of artificial nectar of five different concentrations was placed on extrafloral nectaries (sample sizes appear in *parentheses* below each concentration). After an hour, the ants were counted on the leaf surfaces (*black bars*, mean ± SEM) and at nectaries (*white bars*, mean ± SEM). Artificial nectar of higher concentrations attracted significantly more ants than lower concentrations ($P < 0.0001$). In addition, there was a significant interaction between concentration and observed location of ants ($P = 0.0045$). At concentrations below 200 µg sugar nectary^{-1} 24 h^{-1}, ants did not preferentially visit leaf surfaces or extrafloral nectaries. However, at concentrations above 200 µg sugar nectary^{-1} 24 h^{-1}, ants preferentially visited extrafloral nectaries (*white bars*)

Discussion

Ant visitation and nectar quality and quantity

The number of ants on leaf surfaces increased when natural nectar production increased and when sugar concentration in the artificial nectar trials increased. Natural nectar production was most often less than 500 µg nectary^{-1} 24 h^{-1} and ranged from no measurable nectar produced in 24 h to 2,400 µg nectary^{-1} 24 h^{-1}. The artificial nectar trials ranged from 0 to 950 µg nectary^{-1} and confirmed the results obtained from observations of natural nectar production: increases in nectar production lead to increases in ant visitation. Even at low nectar levels, 20 µg nectary^{-1}, more ants visited leaf surfaces than when water was presented. Ant visitation continued to increase as sugar concentration increased (Fig. 3), further suggesting that nectar quantity on leaf surfaces influences ant visitation. Other authors have also demonstrated that increases in both natural nectar and artificial nectar can increase ant presence and that an increase in ant presence leads to decreased damage to host plant tissue (Bentley 1976; Bentley 1977; Kost and Heil 2005). Bentley (1977), for example, demonstrated that increased nectar production around flower buds of *Bixa orellana* shrubs attracted more ants during certain periods of flower and fruit development, and that plants with more ants had a higher number of healthy fruits. Koptur (1984) also showed a decrease in herbivore damage when ants were present on *Inga*.

Despite the continued increase in ant visitation with increased artificial nectar, *Inga* plants did not commonly present natural nectar rewards that were as concentrated as our artificial rewards. When higher concentrations of artificial nectar were presented, the number of ants on a leaf increased, however the part of a leaf the ants visited was influenced by the nectar concentration. Ants first visited extrafloral nectaries with sugar rewards before patrolling leaf surfaces (Fig. 5). For moderate and low concentrations of artificial nectar (below 200 µg sugar nectary^{-1}), as many ants or more were on leaf surfaces as were at nectaries. However, when the nectar concentration was above 200 µg sugar nectary^{-1}, ants tended to stay at the extrafloral nectaries instead of patrolling leaf surfaces (Fig. 3). This may be why plants do not commonly produce more than 500 µg sugar nectary^{-1} 24 h^{-1}: at higher nectar

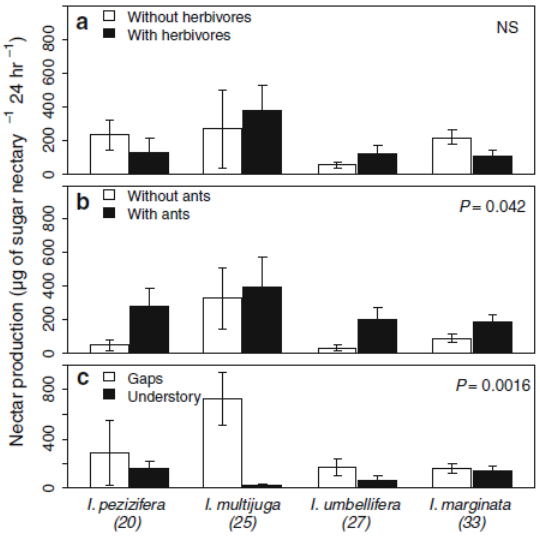

Fig. 4 Nectar production (mean ± SEM) in response to **a** herbivore presence, **b** ant presence, and **c** light. None of the four *Inga* species significantly increased their nectar production when herbivores were present (**a** *NS*). Nectar was induced when ants were present (**b** $P = 0.042$) and when the plants were in gaps (**c** $P = 0.0016$). The P value in each panel (**a**–**c**) represents the value for each of the main effects in an A × B × C ANOVA with species as a blocking variable. There was a significant interaction between light and *Inga* species ($P = 0.0013$). Sample size appears in *parentheses* below each species

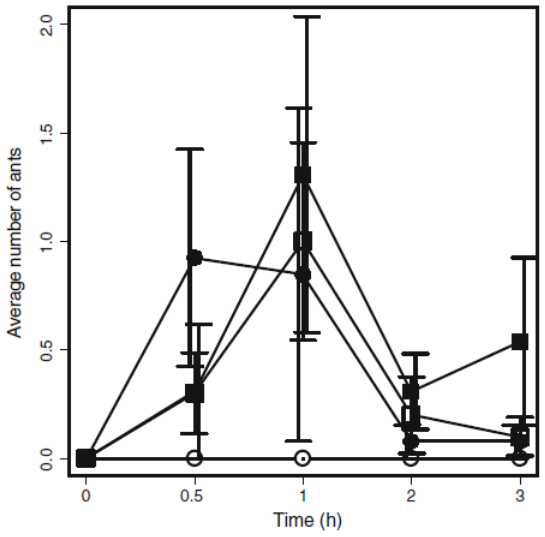

Fig. 5 The timing of ant visits to nectaries and leaf surfaces. Artificial nectar (200 µg µL^{-1}) was placed on two of a leaf's nectaries at time 0. Ants were counted at half an hour, 1 h, 2 h, and at 3 h. Separate observations were made for nectaries with artificial nectar (*filled circles*), nectaries left empty (*open circles*), leaves attached to nectaries with nectar (*filled squares*) and leaves attached to nectaries without nectar (*open squares*). Ants first visit nectaries with artificial nectar (0.5 h), then spread out over leaf surfaces (1 h) before departing the leaves (2 h). Ants spent no time at empty nectaries (*open circles*). Standard errors of the mean are offset to make them more visible at each time point

production rates ants would gorge themselves at nectaries instead of patrolling leaves. This suggests that an intermediate level of nectar production may be most beneficial for plants. This is similar to hypothesized floral nectar production strategies in other systems where intermediate levels of nectar production encouraged continued pollinator foraging and enhanced pollen movement (Zimmerman 1988).

Nectar is principally derived from phloem. Phloem components are "filtered" through a layer of cells below the nectary before being presented as nectar. In these layers, sugars and other essential materials such as amino acids are thought to be added, concentrated or removed in order to transform phloem into nectar (Pacini and Nepi 2007). However, we found no significant difference in sugar concentration among *Inga* species. This suggests that rather than change the concentration of their nectar, plants control the volume of the nectar and thus the mass of sugar produced.

Although amino acids have been reported in the extrafloral nectar of *Inga* (Koptur 1994), these were not analyzed in our study. Baker and Baker (1973) suggested that additional components such as amino acids in floral nectar are likely to influence pollinator visitation. Some studies have shown that ants can have preferences for amino acids in nectar and that herbivory can change amino acid concentrations in nectar (Lanza 1988; Lanza et al. 1993; Smith et al. 1990). However, the same studies also showed that ant preferences can be ant-species specific and that not all plant species responded the same when damaged (Lanza 1988; Lanza et al. 1993; Smith et al. 1990). In addition, Gonzalez-Teuber and Heil (2009) showed that amino acids only affected nectar attractiveness when they were at very high concentrations, and that facultative ant partners in ant–plant mutualisms did not have a preference for changes in amino acid composition. Their results suggested that sugar was more influential in facultative ant–plant mutualisms. In *Inga*, ants visit extrafloral nectaries opportunistically and the identity of a visiting ant species may not be the same from day to day. Thus, plants need a general reward to attract the "passerby" of the day. Heil et al. (2005) demonstrated that qualitative differences in nectar sugar composition can attract specific ants, and that sucrose is more attractive than fructose and glucose to generalist ant bodyguards. Given the above observations from the literature—that sucrose, glucose and fructose were the dominant components of *Inga* nectar, and that there was no difference

in the suite of ant-species that visited the different *Inga* species—we hypothesize that sugars were the principal nutritional components that influenced ant behavior.

Induction

Effect of ants

The increase in nectar production by all species in high light or with ants present demonstrates that nectar production is not strictly constitutive, and that plants are capable of changing their investment in nectar in response to biotic and abiotic signals. We hypothesize that both light and ant presence increase nectar production through a source–sink mechanism. We define nectaries as sinks and photosynthate from the phloem as the source. Photosynthate is most likely imported to young leaves from storage tissues (in mature leaves, roots or stems) due to the high demands in young leaves, a lack of sufficient photosynthetic machinery in young leaves, and, in the understory, insufficient light (Kursar and Coley 1992). As ants remove nectar from the nectary, the sink is emptied then refilled by the phloem. In *Macaranga*, plants that had nectar removed regularly with pipettes produced more nectar than plants that had their nectar collected at longer intervals (Heil et al. 2000). In this study, nectaries were isolated from ants for 24 h before nectar collections were made. During this period, nectaries that had received normal ant visitation produced more nectar despite being isolated from ants for 24 h.

Effect of light

Increased nectar production also occurred when plants were in higher light environments, such as light gaps. There was a significant interaction effect between light and plant species ($P = 0.0013$, Table 1), but all plant species had the same trend of increased nectar production in high light (Fig. 4c). Plants in the understory can be limited by light, as typically less than 2% of full sunlight penetrates through to the rainforest understory (Leigh 1999). The increased flow into the extrafloral nectaries when plants are found in the higher light of gaps could be due to the increased photosynthate in the phloem from storage tissue or photosynthesizing leaves. Similar shifts in carbon-based ant rewards have been observed in *Cecropia* plants. In high light, these plants increased their investment in Mullerian bodies, which are predominately glycogen (Folgarait and Davidson 1994). In addition, when *Inga vera* individuals were grown in the light, they received better protection from their mutualistic ant partners, suggesting increased nectar production (Kersch and Fonseca 2005).

While the greater availability of photosynthate stands out as the predominant reason for increased nectar production in high light, it has also been found that on BCI there is a higher abundance of herbivores and predators of herbivores in gaps (Richards and Coley 2007). So investment in ant defense in gaps would be more likely to attract a defender, and it is an environment where more protection is needed.

Effect of herbivores

Herbivore-induced production of secondary metabolites is regulated by jasmonic acid (JA) and the octadecanoid pathway (Arimura et al. 2005). We showed that one species of herbivore does not induce nectar production in the genus *Inga*. This may be because nectar production (1) is constitutive and therefore not under JA pathway regulation, (2) does not respond either to the amount of damage inflicted by the experimental treatment or to the species of herbivore that we used, or (3) does not respond to short-term changes in herbivore presence. With respect to (1), we have shown in this study that nectar production is not constitutive and can in fact be altered by both biotic and abiotic factors. Heil and Silva Bueno (2007) showed that leaves exposed to volatile organic compounds increased their nectar production through JA signaling. Additionally, Heil et al. (2001) showed that extrafloral nectar is induced by exogenous JA and that this increased ant visitation in *Macaranga tanarius*. This suggests that nectar production can be controlled by JA and is not constitutive in *Inga*.

With respect to (2) above, the larval gelechiids used in this study are the most common herbivores on *Inga* on BCI (Kursar et al. 2006), and, in the plus-herbivore treatment of the current study, they consumed 21% of a young leaf on average. Karban and Baldwin (1997) reported that many other genera show induced responses to "minute" amounts of damage, often well below 20% of the leaf area. These data suggest that if nectar production in the genus *Inga* is responsive to herbivore damage, the experimental treatment was sufficient to elicit an induced response and that an appropriate herbivore was used. However, gelechiid caterpillars do build leaf "tents" to hide from predators. Therefore, because more ant bodyguards may not be more effective against these concealed enemies, this herbivore may not induce nectar production and consequently ant protection.

The final hypothesis, (3) above, suggests that plants would not benefit from relaxing their defenses because herbivore pressure in tropical habitats is high and herbivore-free periods are brief. That is, because herbivore pressure in tropical forests does not vary between high and low periods as in temperate habitats, there may not be a benefit for defenses to respond to short-term changes in herbivore presence on individual plants. The pattern of induced nectar production in temperate plants but not in

tropical plants has been suggested by Koptur (1989), who showed induced nectar production in a temperate annual (*Vicia sativa*) but not in tropical perennial plants (*Ipomoea carnea*, *Inga brenesii*, and *Inga punctata*). While these results support the proposed hypothesis, Koptur points out that the difference could be due to the plant origin (i.e., temperate or tropical) or to growth form (i.e., perennial or annual), and that further research needs to be pursued. Other authors have shown that defenses can be induced in temperate perennial plants (Berenbaum and Zangerl 1994; Bjorkman et al. 2008; Dalin and Bjorkman 2003; Darrow and Bowers 1999; Litvak and Monson 1998; Ness 2003; Scutareanu et al. 2003; Shiojiri and Karban 2006; Wold and Marquis 1997; Wooley et al. 2007), which suggests that the differences found in this study are due to the selective pressures associated with a plant's native habitat and not to its growth form.

Currently, plant–herbivore defense theory emphasizes the advantages of induction. We suggest, however, that induction in response to herbivores may not be adaptive for all environments. The lack of evidence supporting induced defenses in tropical plants suggests that the selective forces are different across a latitudinal gradient and that selection favors constitutive defenses in tropical plants. Furthermore, it suggests that constitutive defenses are adaptive under certain conditions and not necessarily a relic of an ancestral state.

In contrast to the predictably high risk of herbivore attack to young leaves, ant visitation in a facultative mutualism is more variable. If ants are present, it is adaptive to invest in nectar. However, if ants are not present, then the cost of nectar production would not be balanced by the benefits of protection. Thus, we suggest that selection has favored plastic responses of nectar production to ants but not herbivores in tropical forests.

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References

Apple JL, Feener DH (2001) Ant visitation of extrafloral nectaries of *Passiflora*: the effects of nectary attributes and ant behavior on patterns in facultative ant–plant mutualisms. Oecologia 127:409–416

Arimura G, Kost C, Boland W (2005) Herbivore-induced, indirect plant defences. Biochim Biophys Acta 1734:91–111

Baker HG, Baker I (1973) Amino-acids in nectar and their evolutionary significance. Nature 241:543–545

Baker HG, Baker I (1982) Chemical constituents of nectar in relation to pollination mechanisms and phyologeny. In: Nitecki MH (ed) Biochemical aspects of evolutionary biology. University of Chicago Press, Chicago, pp 131–171

Baker HG, Baker I (1983) Floral nectar sugar constituents in relation to pollinator type. In: Jones CE, Little RJ (eds) Handbook of experimental pollination biology. Scientific and Academic Editions, New York, pp 117–141

Bentley BL (1976) Plants bearing extrafloral nectaries and the associated ant community: interhabitat differences in the reduction of herbivore damage. Ecology 57:815–820

Bentley BL (1977) The protective function of ants visiting the extrafloral nectaries of *Bixa orellana* (Bixaceae). J Ecol 65:27–38

Berenbaum MR, Zangerl AR (1994) Costs of inducible defense: protein limitation, growth, and detoxification in parsnip webworms. Ecology 75:2311–2317

Bjorkman C, Dalin P, Ahrne K (2008) Leaf trichome responses to herbivory in willows: induction, relaxation and costs. N Phytol 179:176–184

Bluthgen N, Gottsberger G, Fiedler K (2004) Sugar and amino acid composition of ant-attended nectar and honeydew sources from an Australian rainforest. Austral Ecol 29:418–429

Boege K (2004) Induced responses in three tropical dry forest plant species—direct and indirect effects on herbivory. Oikos 107:541–548

Brenes-Arguedas T, Horton MW, Coley PD, Lokvam J, Waddell RA, Meizoso-O'Meara BE, Kursar TA (2006) Contrasting mechanisms of secondary metabolite accumulation during leaf development in two tropical tree species with different leaf expansion strategies. Oecologia 149:91–100

Bronstein JL, Alarcon R, Geber M (2006) The evolution of plant-insect mutualisms. N Phytol 172:412–428

Coley PD, Aide TM (1991) Comparisons of herbivory and plant defenses in temperate and tropical broad-leaved forests. In: Price PW, Lewinsohn TM, Fernandes GW, Benson WW (eds) Plant–animal interactions: evolutionary ecology in tropical and temperate regions. Wiley, New York, pp 25–49

Coley PD, Barone JA (1996) Herbivory and plant defenses in tropical forests. Annu Rev Ecol Syst 27:305–335

Coley PD, Kursar TA (1996) Anti-herbivore defenses of young tropical leaves: physiological constraints and ecological trade-offs. In: Mulkey SS, Chazdon RL, Smith AP (eds) Tropical forest plant ecophysiology. Chapman & Hall, New York, pp 305–336

Coley PD, Lokvam J, Rudolph K, Bromberg K, Sackett TE, Wright L, Brenes-Arguedas T, Dvorett D, Ring S, Clark A, Baptiste C, Pennington RT, Kursar TA (2005) Divergent defensive strategies of young leaves in two species of *Inga*. Ecology 86:2633–2643

Croat TB (1978) Flora of Barro Colorado Island. Stanford University Press, Stanford

Dalin P, Bjorkman C (2003) Adult beetle grazing induces willow trichome defence against subsequent larval feeding. Oecologia 134:112–118

Darrow K, Bowers MD (1999) Effects of herbivore damage and nutrient level on induction of iridoid glycosides in *Plantago lanceolata*. J Chem Ecol 25:1427–1440

Folgarait PJ, Davidson DW (1994) Antiherbivore defenses of myrmecophytic *Cecropia* under different light regimes. Oikos 71:305–320

Gonzalez-Teuber M, Heil M (2009) The role of extrafloral nectar amino acids for the preferences of facultative and obligate ant mutualists. J Chem Ecol 35:459–468

Heil M, McKey D (2003) Protective ant–plant interactions as model systems in ecological and evolutionary research. Annu Rev Ecol Evol Syst 34:425–453

Heil M, Silva Bueno JC (2007) Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. Proc Natl Acad Sci USA 104:5467–5472

Heil M, Fiala B, Baumann B, Linsenmair KE (2000) Temporal, spatial and biotic variations in extrafloral nectar secretion by *Macaranga tanarius*. Funct Ecol 14:749–757

Heil M, Koch T, Hilpert A, Fiala B, Boland W, Linsenmair KE (2001) Extrafloral nectar production of the ant-associated plant, *Macaranga tanarius*, is an induced, indirect, defensive response elicited by jasmonic acid. Proc Natl Acad Sci USA 98:1083–1088

Heil M, Rattke J, Boland W (2005) Post secretory hydrolysis of nectar sucrose and specialization in ant–plant mutualism. Science 308:560–563

Herms DA, Mattson WJ (1992) The dilemma of plants: to grow or defend. Q Rev Biol 67:283–335

Holdridge L, Grenke W, Hatheway W, Liang T, Tosi J (1971) Forest environments in tropical life zones: a pilot study. Pergamon, Oxford

Karban R, Baldwin IT (1997) Induced responses to herbivory. University of Chicago Press, Chicago

Karban R, Agrawal AA, Thaler JS, Adler LS (1999) Induced plant responses and information content about risk of herbivory. Trends Ecol Evol 14:443–447

Kersch MF, Fonseca CR (2005) Abiotic factors and the conditional outcome of an ant–plant mutualism. Ecology 86:2117–2126

Koptur S (1984) Experimental evidence for defense of *Inga* (Mimosoideae) saplings by ants. Ecology 65:1787–1793

Koptur S (1985) Alternative defenses against herbivores in *Inga* (Fabaceae: Mimosoideae) over an elevational gradient. Ecology 66:1639–1650

Koptur S (1989) Is extrafloral nectar production an inducible defense? In: Bock JH, Linhart YB (eds) The evolutionary ecology of plants. Westview, Boulder, pp 323–339

Koptur S (1992) Extrafloral nectary-mediated interactions between insects and plants. In: Bernays E (ed) Insect–plant interactions, vol 4. CRC Press, Boca Raton, pp 81–129

Koptur S (1994) Floral and extrafloral nectars of Costa Rican *Inga* trees: a comparison of their constituents and composition. Biotropica 26:276–284

Koptur S (2005) Nectar as fuel for plant protectors. In: Wackers FL, PCJv Rijn, Bruin J (eds) Plant-provided food for carnivorous insects: a protective mutualism and its applications. Cambridge University Press, New York, pp 75–108

Kost C, Heil M (2005) Increased availability of extrafloral nectar reduces herbivory in Lima bean plants (*Phaseolus lunatus*, Fabaceae). Basic Appl Ecol 6:237–248

Kursar TA, Coley PD (1992) Delayed development of the photosynthetic apparatus in tropical rain forest species. Funct Ecol 6:411–422

Kursar TA, Coley PD (2003) Convergence in defense syndromes of young leaves in tropical rainforests. Biochem Syst Ecol 31:929–949

Kursar TA, Wolfe BT, Epps MJ, Coley PD (2006) Food quality, competition, and parasitism influence feeding preference in a neotropical lepidopteran. Ecology 87:3058–3069

Lanza J (1988) Ant preferences for *Passiflora* nectar mimics that contain amino acids. Biotropica 20:341–344

Lanza J, Vargo EL, Pulim S, Yu Zong C (1993) Preferences of the fire ants *Solenopsis invicta* and *S.* geminata (Hymenoptera: Formicidae) for amino acid and sugar components of extrafloral nectars. Environ Entomol 22:411–417

Leigh EG (1999) Tropical forest ecology: a view from Barro Colorado Island. Oxford University Press, Oxford

Litvak ME, Monson RK (1998) Patterns of induced and constitutive monoterpene production in conifer needles in relation to insect herbivory. Oecologia 114:531–540

Lokvam J, Kursar TA (2005) Divergence in structure and activity of phenolic defenses in young leaves of two co-occurring *Inga* species. J Chem Ecol 31:2563–2580

Lokvam J, Coley PD, Kursar TA (2004) Cinnamoyl glucosides of catechin and dimeric procyanidins from young leaves of *Inga umbellifera* (Fabaceae). Phytochemistry 65:351–358

Lokvam J, Brenes-Arguedas T, Lee JS, Coley PD, Kursar TA (2006) Allelochemic function for a primary metabolite: the case of L-tyrosine hyper-production in *Inga umbellifera* (Fabaceae). Am J Bot 93:1109–1115

Ness JH (2003) *Catalpa bignonioides* alters extrafloral nectar production after herbivory and attracts ant bodyguards. Oecologia 134:210–218

Pacini E, Nepi M (2007) Nectar production and presentation. In: Nicolson SW, Nepi M, Pacini E (eds) Nectaries and nectar. Springer, Dordrecht, pp 167–214

Richards LA, Coley PD (2007) Seasonal and habitat differences affect the impact of food and predation on herbivores: a comparison between gaps and understory of a tropical forest. Oikos 116:31–40

Richards LA, Windsor DM (2007) Seasonal variation of arthropod abundance in gaps and the understorey of a lowland moist forest in Panama. J Trop Ecol 23:169–176

Rohde S, Wahl M (2008) Antifeeding defense in baltic macroalgae: induction by direct grazing versus waterborne cues. J Phycol 44:85–90

Rudgers JA (2004) Enemies of herbivores can shape plant traits: selection in a facultative ant–plant mutualism. Ecology 85:192–205

Rudgers JA, Gardener MC (2004) Extrafloral nectar as a resource mediating multispecies interactions. Ecology 85:1495–1502

Schupp EW, Feener DH (1991) Phylogeny, lifeform, and habitat dependence of ant-defended plants in a Panamanian forest. In: Huxley CR, Cutler DF (eds) Ant–plant interactions. Oxford University Press, New York

Scutareanu P, Bruin J, Posthumus MA, Drukker B (2003) Constitutive and herbivore-induced volatiles in pear, alder and hawthorn trees. Chemoecology 13:63–74

Shiojiri K, Karban R (2006) Plant age, communication, and resistance to herbivores: young sagebrush plants are better emitters and receivers. Oecologia 149:214–220

Smith LL, Lanza J, Garon CS (1990) Amino acid concentrations in extrafloral nectar of *Impatiens sultani* increase after simulated herbivory. Ecology 71:107–115

Stamp N (2003) Out of the quagmire of plant defense hypotheses. Q Rev Biol 78:23–55

Wold EN, Marquis RJ (1997) Induced defense in white oak: effects on herbivores and consequences for the plant. Ecology 78:1356–1369

Wooley SC, Donaldson JR, Gusse AC, Lindroth RL, Stevens MT (2007) Extrafloral nectaries in aspen (*Populus tremuloides*): heritable genetic variation and herbivore-induced expression. Ann Bot 100:1337–1346

Zimmerman M (1988) Nectar production, flowering phenology, and strategies for pollination. In: Doust JL, Doust LL (eds) Plant reproductive ecology: patterns and strategies. Oxford University Press, Oxford, pp 157–178

CHAPTER 3

TRADE-OFFS BETWEEN CONSTITUTIVE AND INDUCED RESPONSES ARE PREDICTED BY THE PROBABILITY OF ATTACK BY NATURAL INSECT HERBIVORES IN THE NEOTROPICAL TREE GENUS *INGA*

Abstract

Induced defenses are thought to be a cost-saving antiherbivore defense strategy for plants. Over the past three decades, empirical evidence has supported this hypothesis and has demonstrated a diversity of inducible defenses. In addition, there have been substantial advancements in our understanding of the molecular mechanisms that regulate induction. In particular, the signaling hormone jasmonic acid has been shown to upregulate antiherbivore defenses. However, all of our understanding of induced defenses is from temperate and agricultural systems. It remains unknown to what extent tropical plants, which experience far greater herbivore pressure, can induce their defenses. In this study, we used natural herbivores to induce three toxic secondary metabolites in the Neotropical tree genus *Inga*. We gravimetrically quantified phenolics, saponins, and tyrosine and used HPLC-MS to quantify jasmonic acid in damaged and undamaged plants. Saponins were not induced, but phenolics were upregulated in species that have fast-expanding young leaves. Tyrosine was induced to a much greater extent, presumably because it is metabolically more labile. We also demonstrated a trade-off

between induced and constitutive defenses based on the probability of attack on young leaf tissue. While two of the four *Inga* species examined in this study could induce their defenses, it was by a relatively small degree compared to other studies. Furthermore, there was no significant induction of jasmonic acid. Therefore, we suggest that the chemical defenses of tropical young leaves are canalized and constitutively expressed. In addition, these results suggest that the divergent defenses seen in the young leaves of other studies are indeed species-level traits and not plastic responses.

Introduction

Nearly half of the approximately one million described insect species are herbivorous making plant-herbivore interactions one of the most common macroscopic interactions on the planet (Schoonhoven et al. 2005). At the center of these interactions are the defenses that plants express and the strategies that herbivores use to circumvent these defenses. Despite the prevalence of these interactions and their importance in shaping biodiversity and structuring communities, there is still a debate about the adaptive significance of different defense strategies, especially with respect to induced versus constitutive defenses.

Constitutive defenses are expressed constantly regardless of herbivore presence and therefore plants must continually invest in these defenses. In contrast, induced defenses are only expressed in response to herbivore presence (Karban and Baldwin 1997). When herbivores are absent, these plants grow and reproduce, but in the presence of herbivores they reallocate a portion of their resources into defense. Herbivore damage activates the jasmonic acid pathway, the primary phytohormone that regulates induced

responses, which signals the up-regulation of chemistry, physical traits, and predator rewards (Koo and Howe 2009).

The induced resistance hypothesis predicts induced defenses to be adaptive only when herbivory is variable and predictable and when defenses effectively reduce herbivory. In contrast, the hypothesis predicts constitutive defenses to be adaptive when the probability of herbivore damage is constantly high or if the defense is cheap to produce (Figure 3.1, Karban and Baldwin 1997). A large body of research has shown the existence of induction and its relationship with variable herbivore pressure. In contrast, there is little empirical evidence for the adaptive significance of constitutive defenses under high, constant herbivore pressure (Zangerl and Rutledge 1996).

Most tests of the induced resistance hypothesis have focused on temperate ecosystems that typically experience variable herbivore pressure within a growing season. In contrast, tropical plants experience greater herbivore pressure and invest more in defense relative to temperate plants (Coley and Barone 1996). Herbivory is particularly high on young tropical leaves. Herbivores grow twice as fast and suffer lower predation rates on young, expanding, tropical leaves, which are inherently soft and full of nitrogen and are therefore a highly sought-after food resource (Kursar et al. 2006). Once mature, tropical leaves quickly become a tougher, less nutritious food source for insect herbivores (Kursar and Coley 2003). Consequently, during the average two year lifespan of an understory tropical leaf, over 70% of its lifetime herbivore damage (ca 30% of the total leaf area) occurs within the first month while it is expanding (Coley and Aide 1991, Kursar and Coley 2003). Under conditions of consistently high herbivore pressure, the induced resistance hypothesis predicts that constitutive defenses would be more adaptive

than induced defenses. However, few published studies test for induced versus constitutive defenses in tropical forests (Boege 2004, Bixenmann et al. 2011).

In this paper we examine the effects of experimentally manipulated herbivore and ant presence on the concentration of secondary metabolites in four Neotropical tree species in the genus *Inga* (Fabaceae) on Barro Colorado Island, Panama. At the genus level, *Inga* has a diverse array of chemical and phenological defenses (Lokvam et al. 2004, Coley et al. 2005, Lokvam and Kursar 2005, Brenes-Arguedas et al. 2006, Lokvam et al. 2006). Chemical defenses are toxic to herbivores and phenological defenses, such as rapid leaf expansion, allow the leaves to spend less time in the vulnerable young leaf state (Coley et al. 2005). Most species of *Inga* also have extrafloral nectaries that produce nectar and use ant protection to varying degrees (Koptur 1984, 1985, Brenes-Arguedas et al. 2006, Bixenmann et al. 2011). Extrafloral nectaries in *Inga* are located on the rachis between leaflets pairs and only secrete nectar when the leaves are young and expanding. We manipulated herbivore presence and ant presence to determine if *Inga* relies on constitutive or induced defenses.

Methods

Field research was conducted on Barro Colorado Island (BCI), Panama from May to November (rainy season) 2007 and 2008. BCI is located in the Panama Canal (9°N 80°W) and is administrated by the Smithsonian Tropical Research Institute. The island is a tropical moist lowland forest that experiences a 4-month dry season (January to April) (Holdridge et al. 1971, Croat 1978, Leigh 1999). For *Inga*, most young leaves are produced during the rainy season (Coley and Kursar pers. observ.). This is also the

season that herbivores and their predators are most abundant in both gaps and understories (Richards and Windsor 2007).

Field experiment

To assess if herbivores could induce defenses in young leaves, we randomly assigned plants along the trails of BCI to either an herbivore-damage treatment (plus-caterpillar) or an herbivore-free treatment in which herbivores were removed and they received no herbivore damage (minus-caterpillar). Because mechanical wounding alone does not mimic herbivore damage (Arimura et al. 2005), natural herbivores were used to test for an induction response in young leaves of four species of *Inga*: *I. marginata* (n=33), *I. pezizifera* (n=20), *I. multijuga* (n=25), and *I. umbellifera* (n=27). The most common herbivore on *Inga* species on BCI is a black-headed leaf roller (Gelechiidae, species not identified; Kursar et al. 2006). Second and third instar gelechiids were collected from non-focal *Inga* plants and moved to plus-herbivore plants.

To test if ants affect expression of chemical defenses by plants, individual plants in each of the two herbivore treatments were assigned to either a treatment with normal ant visitation to leaves (plus-ants) or to a treatment where ant access to leaves was restricted by the addition of a sticky barrier (Tanglefoot) on the branch (minus-ants). In addition, plants were evenly sampled in both gaps and in the understory to determine the effect of light level and other microclimate differences on chemical defenses. These three factors (canopy, herbivore, and ant treatment) were fully crossed for each species and an individual plant only experienced one level of each treatment.

All of the treatments were applied to the young leaves of saplings between 1 and 4 meters tall. Plants were incorporated into the experiment before their young leaves

reached 15% of their average adult leaf area. If there was any preexisting damage on the young leaves, plants were not included and plants were not reused. Treatments were maintained until leaves reached 80% of their adult size (the size when the majority of herbivory has occurred and defense chemicals are high; Kursar and Coley 2003) or until over half of their leaf tissue was damaged. At these terminal time points, leaves were collected for laboratory analysis. Leaves were clipped from the tree and a subsample of each leaf was placed onto ice immediately for jasmonic acid analysis and transferred into a -80° C freezer within 5 hours of collection. The remaining leaf subsample was placed in a paper envelope in the field and within 5 hours was vacuum dried and then stored at -20° C until chemical analysis.

Chemical analysis

Two classes of chemicals (phenolics and saponins) and one amino acid (tyrosine) were extracted and quantified. These chemicals were selected based on their toxicity to herbivores in previous feeding trials using extracts of *Inga* species and other reports on these compounds (Potter and Kimmerer 1989, Agrell et al. 2003, Coley et al. 2005). Secondary metabolites were extracted, separated and quantified gravimetrically. For *I. marginata*, *I. multijuga,* and *I. pezizifera*, 70-80 mg of sample were homogenized using grinding beads in a 1 mL Nunc Cryo Tube™ and a Wig-l-bug® mixer at 46 Hz for a total of 3 minutes. The grinding beads were removed and 1mL of 80% ethanol was added and mixed. Samples were then centrifuged for 10 minutes at 9055 RCF and 5° C. The supernatant was retained and the extraction was repeated a total of five times with 80% ethanol. The same process was repeated twice with 70% acetone and all collected

supernatants were combined. Pellet and extract were dried under nitrogen, then under a vacuum (0.8 torr) at ambient temperature and weighed.

To remove lipids, 3 mL of 60% methanol and 3 mL of hexane were added to each extract. Vials were shaken and allowed to settle. Once two distinct layers formed, the lipid-containing hexane layer was removed and placed in a preweighed vial. Next, 3 mL of hexanes were added and the separation was repeated for a total of five times. Both the polar organic fraction and lipids were dried under nitrogen and then under a vacuum (0.8 torr) at ambient temperature and weighed.

The polar organic fraction was separated on a liquid chromatography column packed with Bakerbond reverse phase octadecylsilane (ODS). Columns were prepared on 10cc syringes plugged with glass wool and filled with 1.9 g of ODS. Water-methanol solutions were used to serially elute the columns as follows: 30mL of 5% MeOH (organic acids), 10 mL of 5% MeOH (blank), 20 mL of 60% MeOH (phenolics), 10 mL of 60% MeOH (blank), 20 mL of 100% MeOH (saponins) and 10 mL of 70% acetone (blank). Each fraction was collected separately into a labeled preweighed vial, dried under nitrogen, and then under a vacuum (0.8 torr). Fractions were quantified gravimetrically and HPLC was used to verify the class of compounds in each fraction. The blank fractions were used to verify separation among fractions, contained less than 5% of the total mass, and were not included in the analyses.

Tyrosine and phenolics in *Inga umbellifera*

Due to the high quantity of tyrosine in *I. umbellifera*, and tyrosine's low solubility, a special extraction protocol was developed. Twenty-five milligrams of dried leaf sample were homogenized as above and extracted in 2 mL of 10% MeOH (final 10%

MeOH solution adjusted to pH=3 with acetic acid) for 20 minutes at 80° C. Samples were centrifuged at 13250 RCF and ambient temperature for 5 minutes. The resulting supernatant was retained and the extraction was repeated. The combined supernatants were separated on preweighed Agilent SampliQ C18 solid phase extraction columns (500 mg ODS). The supernatant was added to the prepared column and washed with an additional 2 mL 10% MeOH (pH 3). The 10% MeOH wash, containing tyrosine, was dried under vacuum (0.8 torr) and redissolved in 20 mL of 10% MeOH. Samples were then separated on a Hitachi LaChrom Elite with an Omnisphere C18 250 x 2.0 mm column isocratically using 10% MeOH/90% HOH with 0.1% formic acid. Tyrosine was detected at 275 nm and quantified based on peak area and external calibration curves. The SampliQ columns were dried and reweighed and the difference was considered to be the mass of the phenolic fraction trapped on the column. *I. umbellifera* does not contain saponins.

Jasmonic acid

Jasmonic acid (JA) was quantified following Wu et al. (2007) and Wang et al. (2007). The frozen JA subsample was homogenized in a 1mL Nunc Cryo Tube™ as above and then extracted with 1mL of ethyl acetate spiked with 400 ng mL^{-1} of deuterated jasmonic acid (JA-D5) (C/D/N Isotopes Inc, Quebec, Canada). The mixture was centrifuged at 13250 RCF at ambient temperature for 5 minutes and the resulting supernatant was collected. The extraction was repeated once more and the two supernatants were combined. The extracts were dried under vacuum at room temperature and redissolved in 0.3mL 40% MeOH. The redissolved solutions were separated on a Hitachi LaChrom Elite HPL C with an Omnisphere C18 250 x 2.0 mm column and

quantified on a LCQ mass spectrometer based on peak area. The ratio of JA to isotopically labeled JA-D5 standard was used to quantify jasmonic acid (ng JA $(g\ FW)^{-1}$).

Because, in the field, we placed the JA subsample directly onto regular ice rather than dry ice, we collected additional leaves from nonfocal individuals of *I. umbellifera* to examine any changes in JA until it was placed in the -80° C freezer. One leaflet from each leaf was placed on dry ice immediately and the remaining leaflets were placed on wet ice. From each leaf, another leaflet was transferred at 1 hour, 5 hours, and the last leaflet was transferred at 8 hours. There was no significant change in JA over the 8-hour period while on regular ice. In fact, the variation among individual leaves was greater than variation due to treatment within a single leaf.

Statistics

Individual plants were treated as units of replication. When a plant had samples from multiple leaves the values for the multiple collections were averaged for an individual plant. Only values from leaves that were in the targeted size range (60-90% of adult size) were used. The proportion of the three chemical defenses (phenolic fraction, saponins fraction, and tyrosine) within a young leaf were arcsine transformed prior to analysis to meet the assumptions of ANOVA. Analysis of variance were run for each defense compound (Table 3.1) and JA using herbivore presence, ant presence, canopy, young-leaf expansion rate, and young leaf size as explanatory variables with an alpha level of 0.05. *Inga* species was used as a blocking variable.

Results

Phenolics

Overall, there was a significant effect of herbivores on phenolics ($F_{1, 84}$=9.88, P<0.01, Figure 3.2a), however, there was a significant interaction between herbivore presence and young leaf expansion rate ($F_{1, 84}$=4.74, P<0.05, Table 3.1). Young leaf expansion rate is a species level trait that describes how a young leaf expands and develops. Species with slow-expanding young leaves expand from buds to their adult size very slowly (2-4 weeks) and have normal chlorophyll development (they appear light green). Species with fast-expanding young leaves spend very little time (< 2 weeks) as young leaves. They are much softer (drooping or hanging rather than being held horizontal), have much higher nitrogen and water content, and have delayed chlorophyll development (they appear white, pink, or red) (Kursar and Coley 2003). Slow-expanding species (*I. multijuga* and *I. pezizifera*) did not induce phenolic compounds whereas fast-expanding species (*I. marginata* and *I. umbellifera*) did. When herbivores were present, *Inga umbellifera* and *I. marginata* had greater phenolic content by 21% and 25%, respectively (Figure 3.2a). In addition, fast-expanding species had significantly higher phenolic content overall ($F_{1, 84}$=15.09, P<0.001), but this result appears to be driven by *I. umbellifera.*

Ant presence and canopy did not induce phenolic compounds in young leaf tissue (Table 3.1), but, younger leaves did have a higher phenolic concentration ($F_{1, 84}$=14.33, P<0.001). Young leaf samples were only collected from two size classes late in leaf expansion: size 3 (50-74% of adult size) and size 4 (75-100% of adult size). For *I.*

multijuga, *I. umbellifera*, and *I. marginata*, phenolic content decreased by 25%, 35% and 20% respectively from size 3 to size 4.

Saponins

The saponins in *I. pezizifera* and *I. marginata* were not induced by herbivore presence (Figure 3.2b) or ant presence (Table 3.1). However, saponin content was 9% (*I. pezizifera*) and 6% (*I. marginata*) higher when plants were found in gaps ($F_{1, 42}$= 81.34, P>0.001) and, as with phenolic compounds, saponin content decreased by 20% from size 3 young leaves to size 4 in *I. marginata* ($F_{1, 42}$= 18.94, P>0.001). *I. multijuga* and *I. umbellifera* did not contain saponins (Figure 3.2b).

Tyrosine

Only *I. umbellifera* had tyrosine in toxic amounts. Tyrosine was induced by 97% when herbivores were present ($F_{1, 20}$=14.45, P<0.01, Figure 3.2c). As with saponins, tyrosine content increased by 148% in gaps ($F_{1, 20}$=34.42, P<0.001) and decreased by 147% from size 3 young leaves to size 4 ($F_{1, 20}$=11.01, P<0.01).

Jasmonic acid

The average jasmonic acid content was significantly different among *Inga* species (*I. marginata* = 850 ng JA (g FW)$^{-1}$; *I. umbellifera* = 829 ng JA (g FW)$^{-1}$; *I. pezizifera* = 596 ng JA (g FW)$^{-1}$; and *I. multijuga* = 1733 ng JA (g FW)$^{-1}$; $F_{1, 38}$=6.45, P<0.01) but was not significantly induced by herbivore presence (Figure 3.3). There appears to be a trend of higher JA content in damaged leaves but there was no significant difference in the omnibus ANOVA or for individual ANOVAs for each species. Additionally, there was

no relationship between JA and ant presence, canopy cover, leaf size, or young-leaf expansion rate.

Discussion

Induction

Phenolic content ranged from 4% to 41% of leaf dry weight (DW) across the four *Inga* species. Herbivory had no effect for the two slow-expanding species, but caused an induced response by 23% in the two fast-expanding species. This extent of induction is low compared to other studies. A few studies have reported a 2.16 to 7.5 fold (i.e. 116% - 650%) induction of phenolics from herbivore or artificial damage (Schultz and Baldwin 1982, Baldwin and Schultz 1983, Eyles et al. 2003, Dutsadee and Nunta 2008), but, other reports suggest 10-60% induction is more common in woody species (Wagner and Evans 1985, Hartley and Firn 1989, Erwin et al. 2001, Baraza et al. 2004, Moreira et al. 2009). In addition, several studies reported no detectible induction of phenolics in woody plant species (Bryant et al. 1991, Massei et al. 2000, Henery et al. 2008, and more). The only comparable data we could find for induction of phenolics in tropical forests were from the mature leaves of three species, two of which were tropical dry forest species, (*Shorea leprosula*, *Croton pseudoniveus*, and *Bursera instabilis*), where phenolics increased by 26-50% (Boege 2004, Massey et al. 2005). Because this is the first report on induced defenses in young leaves from a moist tropical forest, comparing these results is difficult. However, it appears that levels of induction are lower in the present study than in the temperate zone and other tropical plant species. Furthermore, phenolic content in the young leaves of *Inga* is 49% higher when young leaves begin to expand than after being induced by herbivores later in leaf expansion (Bixenmann et al. In Prep). Therefore, it is

unclear that the 20-25% increase in phenolics found in two species late in leaf expansion would be sufficient to reduce herbivory and thus be biologically significant.

Saponins were not induced by herbivores in this study. Agrell et al. (2003) reported that it took 2-4 days postherbivore damage for the saponin content in alfalfa (*Medicago sativa*: Fabaceae) to increase and that the saponin content peaked around 7 days and returned to baseline levels by 14 days. The young leaves of *I. marginata* and *I. pezizifera* (the two saponin-containing species) spent 7 and 14 days, respectively, exposed to herbivores in the expansion stage. If these two species had similar response times to herbivory as *Medicago*, we would have observed an increase of saponins in at least one of the species. Therefore, it appears that saponins are constitutively expressed in the young leaves of these two *Inga* species.

In contrast to phenolics and saponins, tyrosine did show a substantial increase in response to herbivory (97%). The increase of tyrosine throughout development and its postexpansion removal suggest that it is metabolically labile and therefore can be turned over and reused (Lokvam et al. 2006, Bixenmann et al. In Prep). In contrast, phenolics, and particularly condensed tannins are not easily catabolized and recycled (Feeney 1976, Coley et al. 1985). Accumulating an excess of phenolics in the young leaves may not be adaptive as they would persist once the leaf was full size and tough. Toughness is more effective than chemistry at reducing herbivory and thus “extra” phenolics in the mature leaf would no longer be necessary or beneficial. Therefore, it may be that the small amount by which phenolics were induced was due to physiological constraints on catabolism, whereas labile defenses, such as tyrosine, were more easily induced because they were under different physiological trade-offs.

The carbon-nutrient balance hypothesis predicts that plants in high light should increase carbon-based defenses over nitrogen-based defenses (Bryant et al. 1983). Instead, we found that tyrosine (a nitrogen-containing compound) increased by 148% in gaps but phenolics (carbon-based compounds) did not. However, recent work by Barron et al. (2011) has demonstrated that leguminous nitrogen fixation is facultative in *Inga* and that a significant increase in nodulation and fixation occurs in gaps. Thus in gaps, *Inga* may not be N-limited and could afford to invest in a nitrogen-based defense such as tyrosine.

The timing and amount of damage, as well as the identity of the damage agent can have significant impacts on induced responses. The larval gelechiids used in this study are the most common herbivores on *Inga* on BCI (Kursar et al. 2006) and they consumed 21% of a young leaf on average in the plus-herbivore treatment of this study. Karban and Baldwin (1997) reported that many other plant genera showed induced responses to "minute" amounts of damage; often well below 20% of the leaf area. Taken together, these results suggest that our experimental treatment was sufficient to elicit an induced response in the young leaves of *Inga* if the secondary chemistry in the genus is responsive to herbivore damage.

Trade-offs between induced and constitutive defenses

While a trade-off between induced and constitutive defenses may not occur in all systems (Thaler and Karban 1997, Zhang et al. 2009, Rasmann and Agrawal 2011), induction negatively correlates with the probability of attack in many reports (including a meta-analysis of 31 studies), suggesting that this trade-off does exist in many systems (Zangerl and Rutledge 1996, Koricheva et al. 2004, Zhang et al. 2008). In addition,

mathematical models suggest that investment in constitutive versus induced defenses is better predicted by the probability of herbivore damage than by the amount of leaf damage from herbivory (Ito and Sakai 2009).

Because 70% of all herbivore damage in the tropics occurs on young leaves, species that expand quickly reduce the probability that they will be discovered and consumed. Therefore, the optimal defense hypothesis predicts that fast-expanding leaves should invest more in induced defenses and less in constitutive defenses. Indeed, there was a positive relationship between expansion rate and the percent induction of chemical defenses and extrafloral nectar production combined (R^2=0.66, Figure 3.4) (Bixenmann et al. 2011). There was also a negative relationship between expansion rate and investment in constitutive baseline levels of those same defenses (R^2=0.49, Figure 3.4), although the test of significance for this correlation is unreliable with only four data points (Abdel-Megeed 1984). Fast expanders are essentially "escaping" their herbivores by investing more resources in rapid leaf expansion, thereby making them more ephemeral and difficult to find temporally. As a result, herbivore pressure on fast-expanding species is more sporadic, and defenses are only advantageous should the young leaves be discovered by herbivores. In contrast, slow expanders are vulnerable to herbivory for a longer period of time, have a higher probability of herbivore damage, and consequently invest more in constitutive defenses.

Latitudinal trends of induced defenses

Latitudinal trends in herbivore pressure suggest that *Inga* and trees in the aseasonal tropics should rely primarily on phenological and constitutive chemical defenses to reduce herbivory. Consistent with this is the high investment in constitutive

levels of phenolics for *Inga* (8-23%) and other tropical tree species (Coley and Aide 1991). In contrast, temperate plants should invest more in induced defenses than tropical plants (but, see Rasmann and Agrawal 2011). Koptur (1989) showed induced nectar production in a temperate annual (*Vicia sativa*) but not in tropical perennial plants (*Ipomoea carnea*, *Inga brenesii* and *Inga punctata*). While these results support the proposed latitudinal trend, Koptur points out that the difference could be due to the plant origin (i.e. temperate or tropical) or to growth habits (i.e., perennial or annual). Although there are a few examples of trees that do not induce defenses (Henery et al. 2008), there are many more examples of induced defenses in trees and other temperate perennial plants (Haukioja 1991, Berenbaum and Zangerl 1994, Wold and Marquis 1997, Litvak and Monson 1998, Darrow and Bowers 1999, Dalin and Bjorkman 2003, Ness 2003, Scutareanu et al. 2003, Shiojiri and Karban 2006, Wooley et al. 2007, Bjorkman et al. 2008, Eyles et al. 2010). This suggests that differences in defense chemistry are due to the selective pressures associated with a plant's native habitat rather than its growth form (i.e., perennial or annual). With the exception of tyrosine, we observed only a small amount of induction relative to examples from temperate ecosystems.

Jasmonic acid

The JA concentrations for the plus-caterpillar treatments of the four *Inga* species in this study were within induced levels recorded in other studies. However, the JA concentration for undamaged leaves of *Inga* was higher than undamaged leaves in other studies (Wang et al. 2007, Wu et al. 2007). While there was a trend of increased JA content when herbivores were present for all four *Inga* species, there was no overall significant increase in JA signal with herbivory. There are several possible explanations

for why the JA was not induced: 1) JA accumulation was altered by other environmental factors 2) JA was systematically up-regulated by previous damage to the study plant or 3) we missed the short window in which JA was up-regulated.

The JA pathway is susceptible to disruption by herbivores, abiotic factors, or crosstalk with other signal pathways. There is some evidence that herbivores can manipulate the JA response and even decrease baseline defense levels so that previously attacked plants are a better food source (Sarmento et al. 2011). While gelechiid herbivores may have altered the JA response, the fact that the JA levels and defense chemistry did not decrease when herbivores were present suggests that herbivores did not disrupt JA accumulation. Disruptive crosstalk is also possible between the JA pathway and the salicylic acid pathway (SA). The SA pathway is elicited by other biotic factors such as pathogen infections (Bostock 2005) and may be elicited by endophytic fungi, which are common in tropical forests (Arnold et al. 2000, Hawksworth 2001), and could have affected our results. Additionally, JA pathway sensitivity can be increased when exposed to higher levels of UV-B or lowered when exposed to far-red radiation (Demkura et al. 2009, Moreno et al. 2009). However, there was no difference in JA content between plants in gaps or understories for any of the four species in this study.

Given the relatively high levels of JA in undamaged leaves, it is possible that our study trees had been affected by previous interactions with herbivores or by current interactions of neighboring leaves not included in the experiment. However, all neighboring young leaves on a minus-herbivore plant received the same herbivore treatment and had no damage. There is mounting evidence that plants have a “molecular memory” and may be primed by past experience to respond more quickly or more

strongly to future attacks (Gális et al. 2009). However, primed plants generally increase JA pathway sensitivity rather than total JA content (Conrath et al. 2006), suggesting that the high JA baselines that we observed are not due to priming. There is also evidence that damage from previous years (up to 5 years in *Betula*) can affect future investment in antiherbivore defense (Ruuhola et al. 2007). These long-term responses are called delayed induced resistance, in contrast to the rapid induced resistance that we examined in this study, and are beyond the scope of our experimental design. However, if *Inga* did employ delayed induced resistance, we predict that young leaves would still appear to be constitutively defended because the high, constant herbivore pressure would perpetually induce their defenses through delayed induced resistance.

The most parsimonious explanation for a lack of a significant JA induction is that we missed the short spike of JA in response to damage. JA is up-regulated shortly after damage occurs (usually within a few hours) on leaves and then drops back down to baseline levels within a few hours or days (Gális et al. 2009). However, we maintained herbivore treatment from several days to several weeks. Consequently, if there had been a short burst in JA at the beginning of herbivore treatments, we would not have detected it. However, the trend of higher JA in damaged plants of *Inga* species suggests that there may have been an early JA spike that was repressed by the time we collected the leaves.

Conclusion

The induced resistance hypothesis predicts a trade-off between constitutive and induced defenses based on herbivore pressure (Herms and Mattson 1992, Zhang et al. 2008). Because tropical forests are under higher herbivore pressure than temperate forests (Coley and Aide 1991), it is thought that they should rely more on constitutive defenses

(Van Zandt 2008). Here we have demonstrated that, while species in one tropical tree genus can induce their defenses, it is a relatively small increase. Furthermore, we demonstrate a trade-off between induced and constitutive defenses among closely related species based on the probability of attack from herbivores. This is one of a few reports to examine induced defenses in tropical forests and, to our knowledge, is the first to report on induced responses for a wide variety of defensive traits in tropical young leaves.

Variation in defense strategies among tree species may allow coexistence in diverse tropical forests despite similar growth habits, reproductive strategies, and microhabitats. The arms race between trees and their pests/herbivores can exert selective pressure that may drive divergent evolution and may promote coexistence (Becerra 2007, Becerra et al. 2009, Kursar et al. 2009). Implicit in this hypothesis is a genotypic difference in defense traits among closely related, sympatric species. However, because plants are known to be extremely plastic in their responses to the environment it is important to separate species-level variation from phenotypic plasticity (Ballhorn et al. 2011). Here we show that the variation in defense traits among species of *Inga* is far greater than the phenotypic response to changing biotic and abiotic factors. This suggests that divergent defenses within *Inga* are species-level differences and are not the result of plastic responses such as induction. Furthermore, it supports the hypothesis that the divergence in antiherbivore traits and therefore escape from herbivores may be one important mechanism that permits the coexistence of many tree species in tropical forests.

Table 3.1. ANOVA table for phenolics, saponins, and tyrosine. The table includes main effects and interactions. Plant species was used as a blocking variable to remove variation due to different natural history traits. * P<0.05, **P<0.01, ***P<0.001

Factor	Df	Sum Sq	Mean Sq	F-value	P-value
Phenolics					
Herbivore presence	1	0.02185	0.021845	9.8848	< 0.01 **
Ant presence	1	0.002	0.002003	0.9064	0.344
Canopy	1	0.0035	0.003496	1.582	0.212
Young leaf size	1	0.03167	0.031673	14.3316	< 0.001 ***
Young leaf expansion rate	1	0.03337	0.033367	15.0981	< 0.001 ***
***Inga* species (Block)**	2	0.62103	0.310515	140.5052	< 0.001 ***
Herbivore presence X Ant presence	1	0.00313	0.003128	1.4154	0.238
Ant presence X Canopy	1	0.00716	0.007164	3.2414	0.075
Herbivore X Young leaf size	1	0.00273	0.002726	1.2334	0.27
Herbivore presence X Expansion rate	1	0.01048	0.010477	4.7406	< 0.05 *
Ant presence X Expansion rate	1	0.00309	0.003089	1.398	0.24
Young leaf size X Expansion rate	1	0.01718	0.017176	7.7721	< 0.01 **
Young leaf size X *Inga* species	2	0.02055	0.010273	4.6485	< 0.05 *
Ant presence X Canopy X Expansion rate	2	0.01134	0.005668	2.5646	0.083
Herbivore presence X Young leaf size X Expansion Rate	1	0.01813	0.018132	8.2044	< 0.01 **
Canopy X Young leaf size X Expansion rate	2	0.00607	0.003036	1.3736	0.259
Saponins					
Herbivore presence	1	0.000009	0.000009	0.011	0.917
Ant presence	1	0.00205	0.002051	2.4817	0.123
Canopy	1	0.06724	0.067236	81.3444	< 0.001 ***
Young leaf size	1	0.01566	0.01566	18.9462	< 0.001 ***
***Inga* species (Block)**	1	0.27048	0.270483	327.2416	< 0.001 ***
Herbivore presence X Ant presence	1	0.00372	0.003723	4.5043	< 0.05 *
Young leaf size X *Inga* species	1	0.00462	0.004616	5.5841	< 0.05 *
Tyrosine					
Herbivore presence	1	0.03724	0.037235	14.4512	< 0.01 **
Ant presence	1	0.00011	0.000109	0.0423	0.839
Canopy	1	0.08868	0.088677	34.416	< 0.001 ***
Young leaf size	1	0.02836	0.028362	11.0076	< 0.01 **
Herbivore presence X Ant presence	1	0.01415	0.014147	5.4907	< 0.05 *
Herbivore X Young leaf size	1	0.00715	0.007147	2.7738	0.111

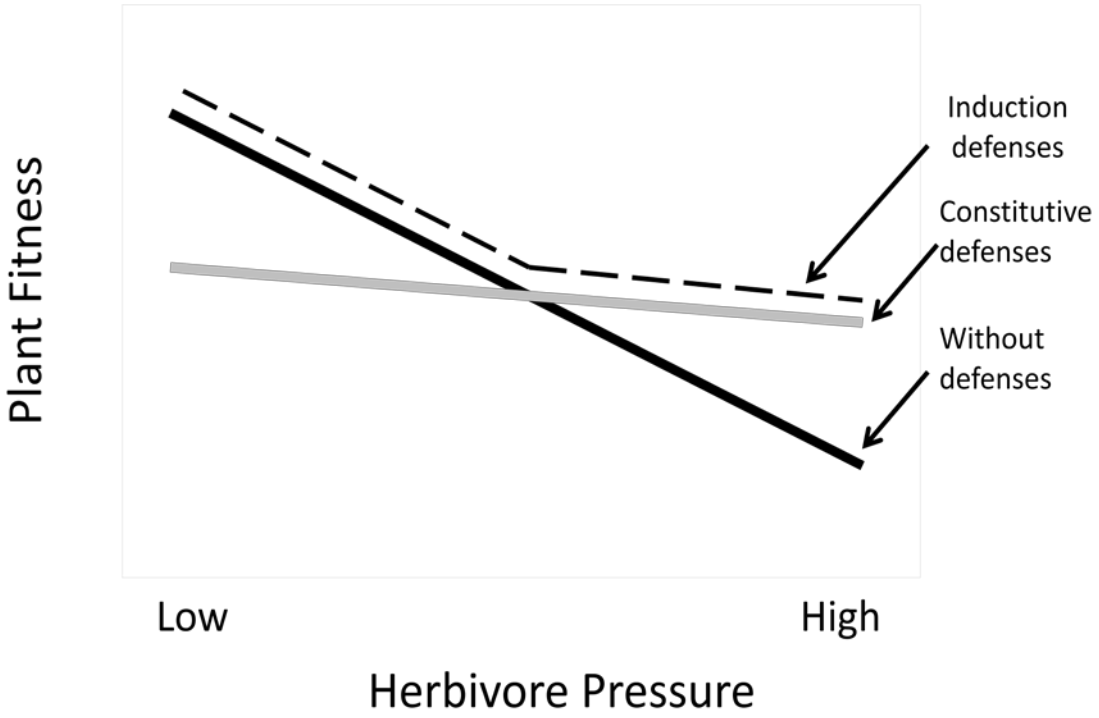

Figure 3.1 Induced resistance hypothesis adapted from Karban et al. (1999). Under low herbivore pressure plants that invest in growth and reproduction (solid black line) are predicted to have a higher fitness than plants that invest in constitutive defenses (solid gray line) because of the assumed costs associated with defense. However, under high herbivore pressure, plants with defenses will have a higher fitness than plants without defenses. Many plants experience intermittent herbivore pressure and benefit from the ability to invest in growth when herbivore pressure is low and induce defenses only when herbivore pressure is high (dashed black line). Tropical young leaves are predicted to be under constant herbivore pressure and therefore to benefit from high levels of constitutive defenses.

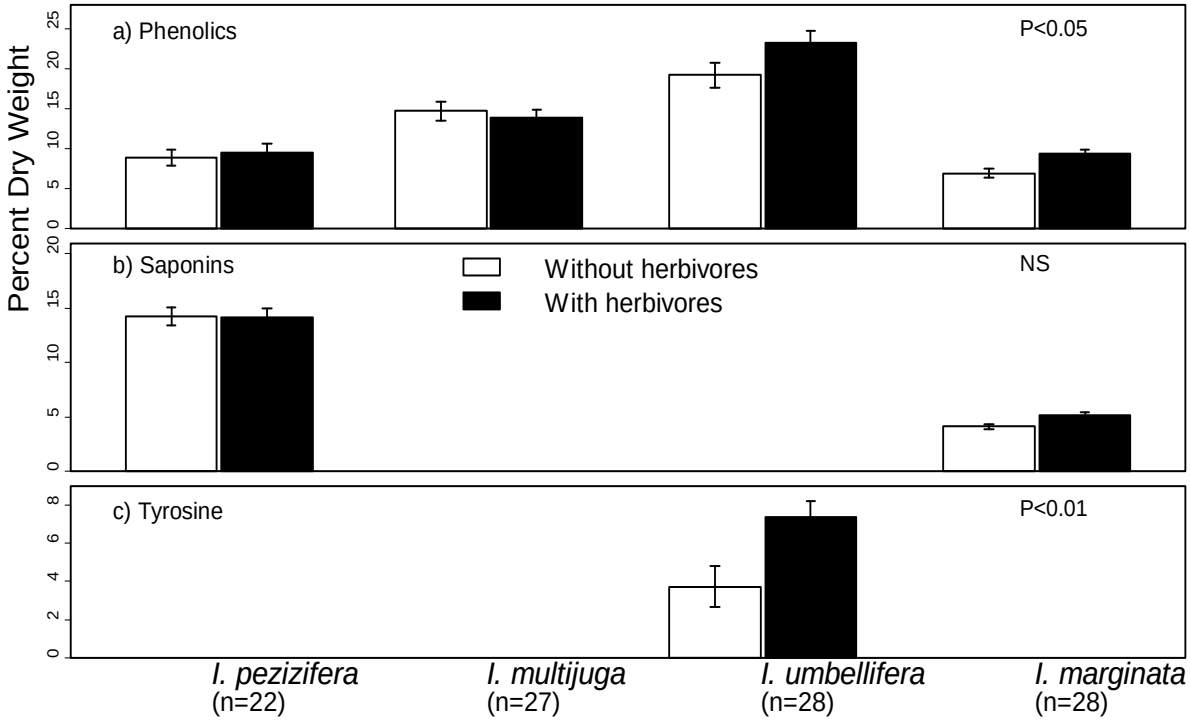

Figure 3.2 Herbivore presence induced the phenolic fraction and tyrosine within the young leaves of four species of *Inga*: *I. pezizifera, I. multijuga, I. umbellifera*, and *I. marginata*. The y-axis is the percent dry weight (mg of fraction mg dry weight^{-1} of leaf) for three principal chemical defenses in *Inga*: (A) phenolics, (B) saponins, and (C) tyrosine. Open bars are individuals from which herbivores were excluded and solid bars are individuals to which herbivores were artificially added. The sample size for each species is included in parenthesis below each species along the x-axis.

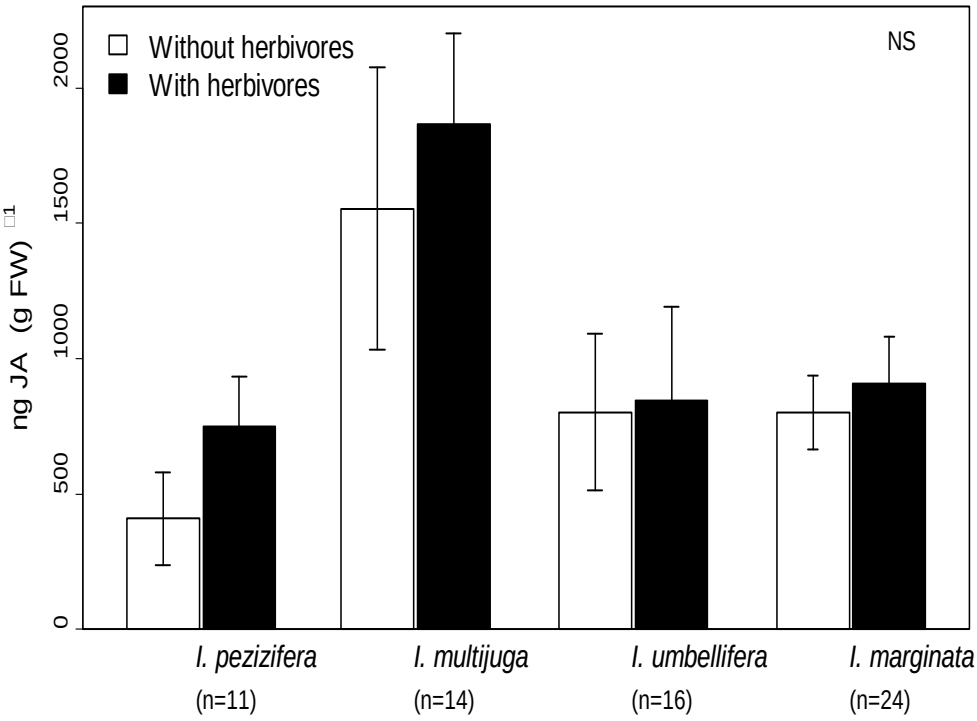

Figure 3.3 For the four *Inga* species (x-axis) there was no significant difference in jasmonic acid content (y-axis) when herbivores were present. There was a significant difference in JA content among *Inga* species ($p<0.01$). Open bars represent means for plants from which herbivores were removed, black bars represent plants to which herbivores were added, and the error bars are standard error of the mean. The sample size for each species is included in parenthesis below each species along the x-axis.

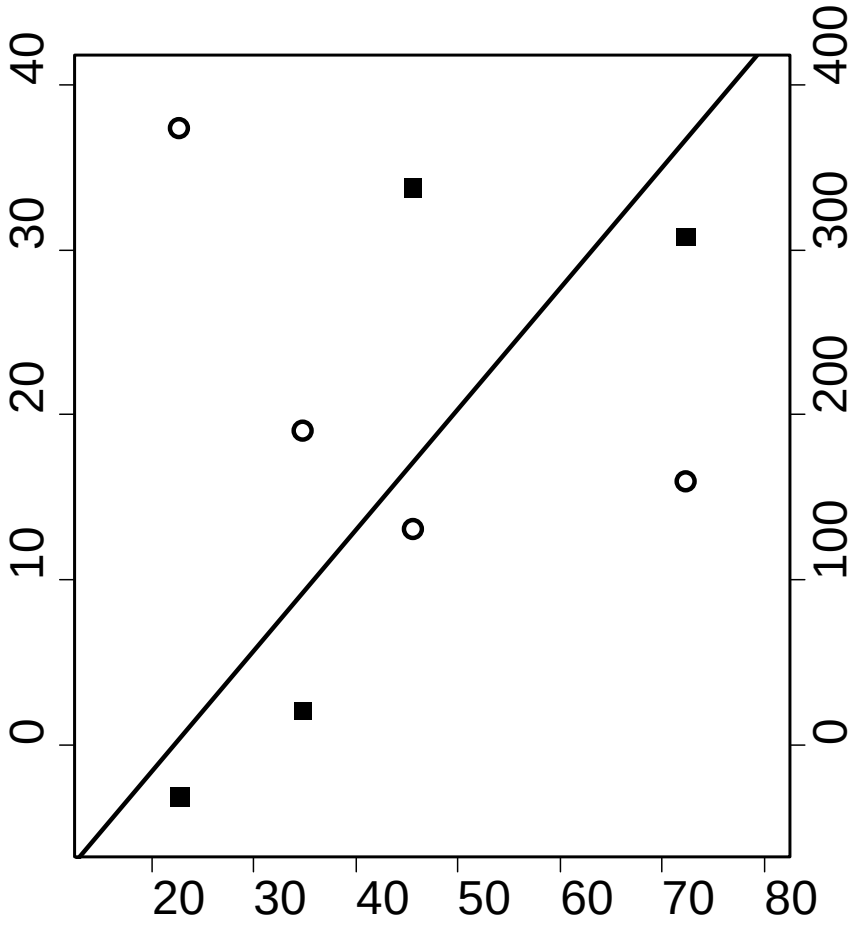

Figure 3.4 Trade-off between investment in constitutive defenses and investment in inducible defenses. Constitutive baseline (open circles and dashed line) is the sum of the percent dry weight of all the chemical defense compounds and extrafloral nectar production rate (µg nectary^{-1} 24 hours^{-1}) for each species: *I. multijuga, I. pezizifera, I. umbellifera,* and *I. marginata.* Percent induced (filled squares and solid line) is the amount of chemical increase from control plants to treatment plants. Expansion rate is the expansion rate of young leaves and calculated as $100*[e^{(\ln(area2/area1)/time)}-1]$.

References

Abdel-Megeed, S. M. 1984. Accuracy of correlation coefficient with limited number of points. The Journal of Experimental Education **52**:188-191.

Agrell, J., W. Oleszek, A. Stochmal, M. Olsen, and P. Anderson. 2003. Herbivore-induced responses in alfalfa (*Medicago sativa*). Journal of Chemical Ecology **29**:303-320.

Arnold, A. E., Z. Maynard, G. S. Gilbert, P. D. Coley, and T. A. Kursar. 2000. Are tropical fungal endophytes hyperdiverse? Ecology Letters **3**:267-274.

Baldwin, I. T. and J. C. Schultz. 1983. Rapid changes in tree leaf chemistry induced by damage: Evidence for communication between plants. Science **221**:277-279.

Ballhorn, D. J., S. Kautz, M. Jensen, I. Schmitt, M. Heil, and A. D. Hegeman. 2011. Genetic and environmental interactions determine plant defences against herbivores. Journal of Ecology **99**:313-326.

Baraza, E., J. M. Gómez, J. A. Hódar, and R. Zamora. 2004. Herbivory has a greater impact in shade than in sun: response of *Quercus pyrenaica* seedlings to multifactorial environmental variation. Canadian Journal of Botany **82**:357-364.

Barron, A. R., D. W. Purves, and L. O. Hedin. 2011. Facultative nitrogen fixation by canopy legumes in a lowland tropical forest. Oecologia **165**:511-520.

Becerra, J. X. 2007. The impact of herbivore-plant coevolution on plant community structure. Proceedings of the National Academy of Sciences **104**:7483-7488.

Becerra, J. X., K. Noge, and D. L. Venable. 2009. Macroevolutionary chemical escalation in an ancient plant-herbivore arms race. Proceedings of the National Academy of Sciences **106**:18062-18066.

Berenbaum, M. R. and A. R. Zangerl. 1994. Costs of inducible defense: Protein limitation, growth, and detoxification in parsnip webworms. Ecology **75**:2311-2317.

Bixenmann, R. J., P. D. Coley, and T. A. Kursar. 2011. Is extrafloral nectar production induced by herbivores or ants in a tropical facultative ant-plant mutualism? Oecologia **165**:417-425.

Bjorkman, C., P. Dalin, and K. Ahrne. 2008. Leaf trichome responses to herbivory in willows: induction, relaxation and costs. New Phytologist **179**:176-184.

Boege, K. 2004. Induced responses in three tropical dry forest plant species - direct and indirect effects on herbivory. Oikos **107**:541-548.

Bostock, R. M. 2005. Signal crosstalk and induced resistance: Straddling the line between cost and benefit. Annual Review of Phytopathology **43**:545-580.

Brenes-Arguedas, T., M. W. Horton, P. D. Coley, J. Lokvam, R. A. Waddell, B. E. Meizoso-O'Meara, and T. A. Kursar. 2006. Contrasting mechanisms of secondary metabolite accumulation during leaf development in two tropical tree species with different leaf expansion strategies. Oecologia **149**:91-100.

Bryant, J. P., F. S. Chapin, and D. R. Klein. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos **40**:357-368.

Bryant, J. P., I. Heitkonig, P. Kuropat, and N. Owen-Smith. 1991. Effects of severe defoliation on the long-term resistance to insect attack and on leaf chemistry in six woody species of the Southern African Savanna. The American Naturalist **137**:50-63.

Coley, P. D. and T. M. Aide. 1991. Comparisons of herbivory and plant defenses in temperate and tropical broad-leaved forests. Pages 25-49 *in* P. W. Price, T. M. Lewinsohn, G. W. Fernandes, and W. W. Benson, editors. Plant-animal interactions: Evolutionary ecology in tropical and temperate regions. John Wiley & Sons, Inc., New York.

Coley, P. D. and J. A. Barone. 1996. Herbivory and plant defenses in tropical forests. Annual Review of Ecology and Systematics **27**:305-335.

Coley, P. D., J. P. Bryant, and F. S. Chapin. 1985. Resource availability and plant antiherbivore defense. Science **230**:895-899.

Coley, P. D., J. Lokvam, K. Rudolph, K. Bromberg, T. E. Sackett, L. Wright, T. Brenes-Arguedas, D. Dvorett, S. Ring, A. Clark, C. Baptiste, R. T. Pennington, and T. A. Kursar. 2005. Divergent defensive strategies of young leaves in two species of *Inga*. Ecology **86**:2633-2643.

Conrath, U., G. J. M. Beckers, V. Flors, P. García-Agustín, G. Jakab, F. Mauch, M. A. Newman, C. M. J. Pieterse, B. Poinssot, M. J. Pozo, A. Pugin, U. Schaffrath, J. Ton, D. Wendehenne, L. Zimmerli, and B. Mauch-Mani. 2006. Priming: Getting ready for battle. Molecular Plant-Microbe Interactions **19**:1062-1071.

Croat, T. B. 1978. Flora of Barro Colorado Island. Stanford University Press, Stanford.

Dalin, P. and C. Bjorkman. 2003. Adult beetle grazing induces willow trichome defence against subsequent larval feeding. Oecologia **134**:112-118.

Darrow, K. and M. D. Bowers. 1999. Effects of herbivore damage and nutrient level on induction of iridoid glycosides in *Plantago lanceolata*. Journal of Chemical Ecology **25**:1427-1440.

Demkura, P. V., G. Abdala, I. T. Baldwin, and C. L. Ballare. 2009. Jasmonate-dependent and -independent pathways mediate specific effects of solar ultraviolet B radiation on leaf phenolics and antiherbivore defense. Plant Physiology **152**:1084-1095.

Dutsadee, C. and C. Nunta. 2008. Induction of peroxidase, scopoletin, phenolic compounds and resistance in *Hevea brasiliensis* by elicitin and a novel protein elicitor purified from *Phytophthora palmivora*. Physiological and Molecular Plant Pathology **72**:179-187.

Erwin, E. A., M. G. Turner, R. L. Lindroth, and W. H. Romme. 2001. Secondary plant compounds in seedling and mature aspen (*Populus tremuloides*) in Yellowstone National Park, Wyoming. The American Midland Naturalist **145**:299-308.

Eyles, A., P. Bonello, R. Ganley, and C. Mohammed. 2010. Induced resistance to pests and pathogens in trees. New Phytologist **185**:893-908.

Eyles, A., N. W. Davies, Z. Q. Yuan, and C. Mohammed. 2003. Host responses to natural infection by *Cytonaema* sp. in the aerial bark of *Eucalyptus globulus*. Forest Pathology **33**:317-331.

Feeney, P. P. 1976. Plant apparency and chemical defense. Pages 1-40 *in* J. Wallace and R. L. Mansell, editors. Biochemical interactions between plants and insects. Recent Advances in Phytochemistry. Plenum Press, New York City.

Gális, I., E. Gaquerel, S. P. Pandey, and I. T. Baldwin. 2009. Molecular mechanisms underlying plant memory in JA-mediated defence responses. Plant, Cell & Environment **32**:617-627.

Hartley, S. E. and R. D. Firn. 1989. Phenolic biosynthesis, leaf damage, and insect herbivory in birch (*Betula pendula*). Journal of Chemical Ecology **15**:275-283.

Haukioja, E. 1991. Induction of Defenses in Trees. Annual Review of Entomology **36**:25-42.

Hawksworth, D. 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycological Research **105**:1422-1432.

Henery, M. L., I. R. Wallis, C. Stone, and W. J. Foley. 2008. Methyl jasmonate does not induce changes in *Eucalyptus grandis* leaves that alter the effect of constitutive defences on larvae of a specialist herbivore. Oecologia **156**:847-859.

Herms, D. A. and W. J. Mattson. 1992. The dilemma of plants: To grow or defend. Quarterly Review of Biology **67**:283-335.

Holdridge, L., W. Grenke, W. Hatheway, T. Liang, and J. Tosi. 1971. Forest environments in tropical life zones: A pilot study. Pergamon Press, Oxford.

Ito, K. and S. Sakai. 2009. Optimal defense strategy against herbivory in plants: Conditions selecting for induced defense, constitutive defense, and no-defense. Journal of Theoretical Biology **260**:453-459.

Karban, R., A. A. Agrawal, J. S. Thaler, and L. S. Adler. 1999. Induced plant responses and information content about risk of herbivory. Trends in Ecology & Evolution **14**:443-447.

Karban, R. and I. T. Baldwin. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago, IL.

Koo, A. J. K. and G. A. Howe. 2009. The wound hormone jasmonate. Phytochemistry **70**:1571-1580.

Koptur, S. 1984. Experimental evidence for defense of *Inga* (Mimosoideae) saplings by ants. Ecology **65**:1787-1793.

Koptur, S. 1985. Alternative defenses against herbivores in *Inga* (Fabaceae: Mimosoideae) over an elevational gradient. Ecology **66**:1639-1650.

Koricheva, J., H. Nykanen, and E. Gianoli. 2004. Meta-analysis of trade-offs among plant antiherbivore defenses: are plants jacks-of-all-trades, masters of all? The American Naturalist **163**:E64-75.

Kursar, T. A. and P. D. Coley. 2003. Convergence in defense syndromes of young leaves in tropical rainforests. Biochemical Systematics and Ecology **31**:929-949.

Kursar, T. A., K. G. Dexter, J. Lokvam, R. T. Pennington, J. E. Richardson, M. G. Weber, E. T. Murakami, C. Drake, R. McGregor, and P. D. Coley. 2009. The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus *Inga*. Proceedings of the National Academy of Sciences **106**:18073-18078.

Kursar, T. A., B. T. Wolfe, M. J. Epps, and P. D. Coley. 2006. Food quality, competition, and parasitism influence feeding preference in a Neotropical lepidopteran. Ecology **87**:3058-3069.

Leigh, E. G. 1999. Tropical forest ecology: A view from Barro Colorado Island. Oxford University Press, Oxford.

Litvak, M. E. and R. K. Monson. 1998. Patterns of induced and constitutive monoterpene production in conifer needles in relation to insect herbivory. Oecologia **114**:531-540.

Lokvam, J., T. Brenes-Arguedas, J. S. Lee, P. D. Coley, and T. A. Kursar. 2006. Allelochemic function for a primary metabolite: the case of L-tyrosine hyper-production in *Inga umbellifera* (Fabaceae). American Journal of Botany **93**:1109-1115.

Lokvam, J., P. D. Coley, and T. A. Kursar. 2004. Cinnamoyl glucosides of catechin and dimeric procyanidins from young leaves of *Inga umbellifera* (Fabaceae). Phytochemistry **65**:351-358.

Lokvam, J. and T. A. Kursar. 2005. Divergence in structure and activity of phenolic defenses in young leaves of two co-occurring *Inga* species. Journal of Chemical Ecology **31**:2563-2580.

Massei, G., S. E. Hartley, and P. J. Bacon. 2000. Chemical and morphological variation of Mediterranean woody evergreen species: Do plants respond to ungulate browsing? Journal of Vegetation Science **11**:1-8.

Massey, F. P., M. C. Press, and S. E. Hartley. 2005. Long- and short-term induction of defences in seedlings of *Shores leprosula* (Dipterocarpaceae): Support for the carbon:nutrient balance hypothesis. Journal of Tropical Ecology **21**:195-201.

Moreira, X., L. Sampedro, and R. Zas. 2009. Defensive responses of *Pinus pinaster* seedlings to exogenous application of methyl jasmonate: Concentration effect and systemic response. Environmental and Experimental Botany **67**:94-100.

Moreno, J. E., Y. Tao, J. Chory, and C. L. Ballaré. 2009. Ecological modulation of plant defense via phytochrome control of jasmonate sensitivity. Proceedings of the National Academy of Sciences **106**:4935-4940.

Ness, J. H. 2003. *Catalpa bignonioides* alters extrafloral nectar production after herbivory and attracts ant bodyguards. Oecologia **134**:210-218.

Potter, D. A. and T. W. Kimmerer. 1989. Inhibition of herbivory on young holly leaves: Evidence for the defensive role of saponins. Oecologia **78**:322-329.

Rasmann, S. and A. A. Agrawal. 2011. Latitudinal patterns in plant defense: evolution of cardenolides, their toxicity and induction following herbivory. Ecology Letters.

Richards, L. A. and D. M. Windsor. 2007. Seasonal variation of arthropod abundance in gaps and the understorey of a lowland moist forest in Panama. Journal of Tropical Ecology **23**:169-176.

Ruuhola, T., J.-P. Salminen, S. Haviola, S. Yang, and M. J. Rantala. 2007. Immunological memory of mountain birches: Effects of phenolics on

performance and the autumnal moth depend on herbivory history of trees. Journal of Chemical Ecology **33**:1160-1176.

Sarmento, R. A., F. Lemos, P. M. Bleeker, R. C. Schuurink, A. Pallini, M. G. A. Oliveira, E. R. Lima, M. Kant, M. W. Sabelis, and A. Janssen. 2011. A herbivore that manipulates plant defence. Ecology Letters **14**:229-236.

Schoonhoven, L. M., J. J. A. Van Loon, and M. Dicke. 2005. Insect-plant biology. Oxford University Press, USA.

Schultz, J. C. and I. T. Baldwin. 1982. Oak leaf quality declines in response to defoliation by Gypsy Moth larvae. Science **217**:149-151.

Scutareanu, P., J. Bruin, M. A. Posthumus, and B. Drukker. 2003. Constitutive and herbivore-induced volatiles in pear, alder and hawthorn trees. Chemoecology **13**:63-74.

Shiojiri, K. and R. Karban. 2006. Plant age, communication, and resistance to herbivores: young sagebrush plants are better emitters and receivers. Oecologia **149**:214-220.

Thaler, Jennifer S. and R. Karban. 1997. A phylogenetic reconstruction of constitutive and induced resistance in *Gossypium*. The American Naturalist **149**:1139-1146.

Van Zandt, P. A. 2008. Plant defense, growth, and habitat: A comparative assessment of constitutive and induced resistance. Ecology **88**:1984-1993.

Wagner, M. R. and P. D. Evans. 1985. Defoliation increases nutritional quality and allelochemics of pine seedling. Oecologia **67**:235-237.

Wang, L., R. Halitschke, J.-H. Kang, A. Berg, F. Harnisch, and I. T. Baldwin. 2007. Independently silencing two JAR family members impairs levels of trypsin proteinase inhibitors but not nicotine. Planta **226**:159-167.

Wold, E. N. and R. J. Marquis. 1997. Induced defense in white oak: Effects on herbivores and consequences for the plant. Ecology **78**:1356-1369.

Wooley, S. C., J. R. Donaldson, A. C. Gusse, R. L. Lindroth, and M. T. Stevens. 2007. Extrafloral nectaries in aspen (*Populus tremuloides*): Heritable genetic variation and herbivore-induced expression. Annals of Botany **100**:1337-1346.

Wu, J., C. Hettenhausen, S. Meldau, and I. T. Baldwin. 2007. Herbivory Rapidly Activates MAPK Signaling in Attacked and Unattacked Leaf Regions but Not between Leaves of *Nicotiana attenuata*. Plant Cell **19**:1096-1122.

Zangerl, A. R. and C. E. Rutledge. 1996. The probability of attack and patterns of constitutive and induced defense: A test of optimal defense theory. The American Naturalist **147**:599-608.

Zhang, P.-J., J.-P. Shu, C.-X. Fu, Y. Zhou, Y. Hu, M. P. Zalucki, and S.-S. Liu. 2008. Trade-offs between constitutive and induced resistance in wild crucifers shown by a natural, but not an artificial, elicitor. Oecologia **157**:83-92.

Zhang, P.-J., J.-P. Shu, Z.-Y. Wu, M. Dicke, and S.-S. Liu. 2009. Lack of correlation between constitutive and induced resistance to a herbivore in crucifer plants: real or flawed by experimental methods? Entomologia Experimentalis Et Applicata **131**:58-66.

CHAPTER 4

DEVELOPMENTAL CHANGES IN DIRECT AND INDIRECT DEFENSES IN THE YOUNG LEAVES OF THE NEOTROPICAL TREE GENUS *INGA*

Abstract

Plant fitness is affected by herbivory, and in tropical forests, 70% of herbivore damage occurs on young leaves. Thus, in order to understand the effects of herbivory on tropical plant fitness, it is necessary to understand how tropical young leaves survive the brief, but critical, period of susceptibility. In this study we surveyed three species of *Inga* during young leaf expansion. Three toxic secondary metabolites (phenolics, saponins, and tyrosine), extrafloral nectar production, leaf area and extrafloral nectary area were measured at randomly assigned young leaf sizes. In addition, all defenses were compared for potential trade-offs during leaf expansion. No trade-offs were found, and the concentration of all defenses, except tyrosine, decreased during leaf expansion. We suggest that plants continued to increase phenolic and saponin content but at a rate that resulted in decreasing concentrations. In contrast, tyrosine content per leaf steadily increased such that a constant concentration was maintained regardless of young leaf size. Nectar production remained constant during leaf expansion, but, because young leaf area increased by 10-fold, the investment in extrafloral nectar per leaf area significantly decreased. In addition, nectary area did not change during leaf expansion and therefore the relative size of the nectary significantly decreased during young leaf expansion.

These results demonstrate that the youngest leaves have the highest investment in multiple defenses, most likely because they have the highest nitrogen content and are the most susceptible to a diversity of herbivores.

Introduction

Herbivory has major impacts on plant fitness and indirect effects on the outcomes of other biotic interactions. In the understory of tropical moist forests, over 70% of a leaf's lifetime herbivore damage occurs while it is young and expanding. Because young leaves are inherently less tough and more nutritious than mature leaves, they are particularly attractive to herbivores (Brunt et al. 2006, Yadav et al. 2010). Consequently, tropical plants invest an impressive amount of resources into young leaf defenses. Typically, chemical defenses alone comprise 10-50% of the dry weight of young leaves (Coley and Aide 1991, Coley and Barone 1996, Boege 2004, Massey et al. 2005, Brenes-Arguedas et al. 2006, Lokvam et al. 2006). In addition, they invest in other indirect biotic defenses or physical defenses such as spines and hairs. Once leaves have reached their adult size, they become tough and low in nutrients, such that they are difficult for herbivores to consume. As a consequence, herbivory rates decrease to nearly zero (Kursar and Coley 2003). Therefore, if herbivory affects tropical plant performance, it is important to understand how young leaves survive the brief, but critical, period of susceptibility.

While developmental changes in defenses of tropical leaves have been examined in other studies (Heil et al. 2000, Brenes-Arguedas et al. 2006, Lokvam et al. 2006), few have compared the investment trade-off between direct and indirect defenses. Direct

defenses are produced by a plant and directly impact the performance of an herbivore and reduce future herbivory. Common examples are toxic chemicals or trichomes. In contrast, indirect defenses rely on an external agent to reduce herbivore performance and consequently future herbivore damage (Kost and Heil 2008). Common examples of indirect defenses are volatile organic compounds that attract predators or parasitoids of herbivores and extrafloral nectaries that attract ants that act as bodyguards. The optimal defense hypothesis predicts that plants only invest in a subset of defenses because they are costly and resources are limited (Rhoades 1979). Therefore a trade-off among defenses would exist, which could take place spatially or temporally at different developmental stages. For example, ant bodyguards may be more effective than toxic compounds when leaves are small and easily patrolled, but, less effective than toxic compounds when leaves are large. However, it is also possible that defenses are additive and that the complete suite of different defenses is required to effectively protect a plant against a diversity of potential herbivores.

In this study we examine the relative investment in direct and indirect defenses during young leaf expansion in three Neotropical tree species in the genus *Inga* (Fabaceae) on Barro Colorado Island, Panama. At the genus level, *Inga* has a diverse array of toxic chemical defenses (Lokvam et al. 2004, Coley et al. 2005, Lokvam and Kursar 2005, Brenes-Arguedas et al. 2006, Lokvam et al. 2006). Most species of *Inga* also have extrafloral nectaries that produce nectar and use ant protection to varying degrees (Koptur 1984, 1985, Brenes-Arguedas et al. 2006, Bixenmann et al. 2011). Extrafloral nectaries in *Inga* are located on the rachis between leaflets pairs and only secrete nectar when the leaves are young and expanding. In this study we examined the

investment in extrafloral nectar production and extrafloral nectary structures relative to young leaf area during leaf expansion. In addition, we examined the investment in toxic secondary metabolites and compared the response variables for trade-offs. Recognizing the extensive divergence in the defense traits among species of *Inga*, we compared the ontogenetic changes in defenses for several species.

Methods

Study Site

Field research was conducted on Barro Colorado Island (BCI), Panama from May to July 2007 and 2008. BCI is located in the Panama Canal (9°N 80°W) and is administrated by the Smithsonian Tropical Research Institute. The island is a tropical moist lowland forest that experiences a 4-month dry season (January to April) (Holdridge et al. 1971, Croat 1978, Leigh 1999). For *Inga*, most young leaves are produced during the rainy season (Coley and Kursar pers. observ.). This is also the season that herbivores and their predators are most abundant in both gaps and understories (Richards and Windsor 2007).

Field Survey

The young leaves of three focal *Inga* species (*I. marginata*, *I. multijuga*, and *I. umbellifera*) were selected and monitored along the trails of BCI. One branch per individual tree between 1 and 4 meters tall was sampled. No manipulative treatments were applied to any of the plants, but each focal plant was sampled at a randomly, preassigned young-leaf size and care was taken to sample evenly from plants in light gaps and the understory. Mature and young leaf areas from each focal plant were used to

calculate young-leaf age expressed as percent of mature leaf size (area of young leaf/area of mature leaf). Extrafloral nectar and leaf samples were collected in tandem and were vacuum dried and stored at -20°C until they were prepared for analysis (see below). Individual leaves were not sampled more than once to avoid potential effects of induced defenses on the remaining tissue.

Leaf and extrafloral nectary area

Each time a focal leaf was visited, the leaf was photographed with a scale (Figure 4.1). The leaf was oriented perpendicular to the lens of the camera with the entire leaf blade in the same plane. We used NIH ImageJ (http://www.rsb.info.nih.gov/ij/) to calculate the absolute young leaf area (cm^2), area lost to herbivores (cm^2), and the absolute nectary area (cm^2). In addition, we calculated percent damage (area of herbivore damage/absolute young leaf area), relative extrafloral nectary area (absolute nectary area/ absolute young leaf area), and percent adult size (absolute young leaf area/absolute mature leaf area) by using mature leaves from each focal plant.

Extrafloral nectar production

To collect extrafloral nectar from the young leaves, nectaries were first washed with distilled water to remove accumulated nectar. Then the entire leaf was placed in a plastic bag to prevent rain or insects from removing nectar. We did not use mesh bags because they would not have prevented disturbance by rain. While plastic bags may influence temperature and humidity, the bags were not air tight and samples were collected mostly in the shaded understory and during the rainy season when air temperatures were cooler. In addition, condensation on the inside of the bags was rarely

observed in either habitat (gap or understory), indicating no significant heat difference between the inside and outside of the bag. After 24 hours, extrafloral nectar was collected and its volume measured using microcapillary tubes (initial collection). To collect any residual nectar, one microliter drops of distilled water were placed on nectaries, collected, and added to the initial collection. The nectar was collected into glass GC vials, dried under vacuum and frozen at -50°C until analysis.

An HP 6890 gas chromatograph with a DB-1 capillary column and FID was used to identify and quantify the sugars in the nectar. The majority of the components in the nectar were the sugars fructose, glucose, and sucrose. The dried nectar samples were dissolved in 50µL of pyridine. Due to the polarity of sugars, samples were derivatized by adding 50µL of BSTFA with 1% TCMS to the pyridine solution and allowed to sit for 10 hours. External standards and calibration curves were used to identify and quantify the three sugars in the nectar. The total mass of the three sugars was summed for each sample and corrected by the number of nectaries per leaf over a 24 hour period (µg sugar/ nectary /24 hours) or by the leaf area over a 24 hour period (µg sugar/ cm^2 /24hr).

Secondary metabolite analysis

We quantified two classes of secondary metabolites (phenolics and saponins) that are known to be toxic to herbivores based on previous feeding trials using extracts of *Inga* species and other reports on these compounds (Potter and Kimmerer 1989, Agrell et al. 2003, Coley et al. 2005). Secondary metabolites were extracted, separated and quantified gravimetrically. For *I. marginata* and *I. multijuga*, 70-80mg of sample were homogenized using grinding beads in a 1mL Nunc Cryo Tubes™ and a Wig-l-bug®

mixer at 46Hz for a totally of 3 minutes. The grinding beads were removed and 1mL of 80% ethanol was added and mixed. Samples were then centrifuged for 10 minutes at 9055 RCF and 5°C. The supernatant was retained and the extraction was repeated a total of five times with 80% ethanol. The same process was repeated twice with 70% acetone and all collected supernatants were combined. Pellet and extract were dried under nitrogen, then under a vacuum (0.8 torr) at ambient temperature and weighed.

Three mL of 60% methanol and 3mL of hexane were added to each sample extract vial to remove lipids. Vials were shaken and allowed to settle. Once two distinct layers formed, the lipid containing hexane layer was removed and placed in a preweighed vial, 3mL of hexane were added and the separation was repeated for a total of five times. Both the polar organic fraction and lipids were dried under nitrogen and then under a vacuum (0.8 torr) at ambient temperature and weighed.

The polar organic fraction was separated on a preparatory liquid chromatography column packed with octadecylsilane (ODS). Columns were prepared by using 10cc syringes plugged with glass wool and filled with 1.9g of ODS. Water-methanol solutions were used in the following concentrations: 5% MeOH (organic acids), 60% MeOH (phenolics), and 100% MeOH (saponins). Each fraction was collected separately into a labeled preweighed vial, dried under nitrogen, and then under a vacuum (0.8 torr). Fractions were quantified gravimetrically and HPLC was used to verify the class of compounds in each fraction.

Tyrosine and phenolics in *Inga umbellifera*

Tyrosine is toxic at the high concentrations found in *I. umbellifera* (Lokvam et al. 2006). In addition to its high concentration, the low solubility of tyrosine required a special extraction protocol. First, 25mg of dried leaf sample were homogenized using grinding beads in a 1mL Nunc Cryo Tubes™ and a Wig-l-bug® mixer at 46Hz for a totally of 3 minutes. The homogenized, dried leaf sample was extracted in 2mL of 10% MeOH (acidified with acetic acid to pH=3) for 20 minutes at 80°C. Samples were centrifuged at 13250 RCF and ambient temperature for 5 minutes. The resulting supernatant was retained and the extraction was repeated once more. The combined supernatants were separated on preweighed Agilent SampliQ C18 solid phase extraction columns (500mg ODS). The supernatant was added to the prepared column and washed with an additional 2mL 10% MeOH (pH3). The 10% MeOH wash containing tyrosine was dried under vacuum (0.8 torr) and redissolved in 20mL of 10% MeOH. Samples were then separated on a Hitachi LaChrom Elite with an Omnisphere C18 250 x 2.0mm column with an isocratic gradient of 10% MeOH/90% HOH with 0.1% formic acid. Tyrosine was detected and quantified using a diode array detector and external calibration curves. The SampliQ columns were dried and reweighed and the difference was considered to be the mass of the phenolic fraction trapped on the column. *Inga umbellifera* did not contain saponins

Statistics

We used analysis of covariance (ANCOVA) to test for significance because we had a mix of continuous and categorical data. We conducted separate ANCOVAs for

nectar volume, absolute nectary area, relative nectary area, nectar production per nectary, nectar production per leaf area, percent phenolics, percent saponins, and percent tyrosine. The percent phenolics, percent saponins, and percent tyrosine were arcsine transformed to meet the assumptions of ANCOVA. In addition, absolute nectary area, average nectary area, nectary area per leaf area, and nectar production per leaf area were log transformed to meet the assumptions of ANCOVA. The same explanatory variables (young leaf size, percent damaged, plant species, and canopy cover) were used for each ANCOVA and nonsignificant parameters were removed from each model using the step function in R (R Development Core Team 2009). For tyrosine (in *I. umbellifera*) and saponins (in *I. marginata*), plant species was not used because they were each found in only one species. In addition, Pearson's product-moment correlations were computed for all possible combinations of the response variables.

Results

Extrafloral nectar and nectaries

As young leaves expanded, the nectary area became an increasingly smaller proportion of the total leaf area (Figure 4.2a, $F_{1, 76}$=150.41, R^2=0.68, P<0.001). In addition, there was a difference in the ratio of extrafloral nectary area to young leaf area among species ($F_{2, 76}$=8.48, P<0.001), but, average nectary area was not different among species, nor did it change during leaf expansion (Table 4.1). Similarly, nectar volume (µL/young leaf/24 hours) and nectar production per nectary (µg/nectary/24 hours) did not decrease as leaves expanded (Figure 4.2b), but nectar production per leaf area (µg/cm^2/24 hours) did significantly decrease as leaves expanded (Figure 4.2c, $F_{1, 70}$=34.43, R^2=0.32,

P<0.001). In addition, nectar volume (µL/young leaf/24 hours) was significantly different among species ($F_{2, 67}$=5.98, P<0.01).

Chemical defenses

There was a significant difference in phenolic concentration among species (Figure 4.3a, $F_{2, 50}$=4.82, P<0.05) and phenolic concentrations decreased similarly for all species as leaves expanded (*I. marginata* = 67%, *I. umbellifera* = 37%, and *I. multijuga* = 46%; Figure 4.3a, $F_{1, 50}$=17.38, R^2=0.35, P<0.001). Only *I. marginata* had saponins and the saponin concentration also decreased (51%) as young leaves expanded (Figure 4.3b, $F_{1, 11}$=43.86, R^2=0.50, P<0.001). *Inga umbellifera* was the only species that contained tyrosine and there was no significant relationship between tyrosine and leaf age or any of the other explanatory variables or other defenses (Figure 4.3c). The only significant relationship between response variables was a positive correlation between saponins and phenolics in *I. marginata* (Figure 4.4d; R^2=0.81, P<0.001).

Discussion

Nectaries were a substantial proportion of the youngest leaves and did not change size throughout leaf development. Investment in extrafloral nectaries early rather than late in leaf development could either be a developmental constraint or an adaptive trait. However, given that other tissues such as the rachis and leaflets expand considerably, physiological constraints on expansion of nectaries is unlikely. Most likely, early investment in nectaries is advantageous. The nectaries on *Inga* are large and elevated and require an investment in tissue, suggesting a one-time construction cost that may be balanced by anti-herbivore defense benefits (Elias 1983, Pennington 1997). Extrafloral

nectaries on many other species are much less developed and demonstrate that an investment in a structure is not required to attract ant bodyguards (Elias 1983). However, there are advantages to investing in nectary tissue: 1) nectar is less affected by environmental factors, 2) nectary parenchyma tissue can photosynthesize, 3) nectaries can be conspicuous and therefore act as a visual target to attract ants (Mondor and Addicott 2003, Pacini and Nepi 2007). While we do not know the adaptive significance of nectary tissue in *Inga*, the presence of this nonessential structure suggests an advantage to producing the tissue. Furthermore, an early one-time investment in the nectary structure may be adaptive when leaf tissue is the most susceptible, has the highest potential value, and requires the most defense.

Since nectar concentration (µg sugar/µL nectar), nectar volume (µL/ leaf/24 hours), and nectar production per leaf (µg/nectary/24 hours) did not significantly change during young leaf expansion, nectar production per leaf area (µg/cm^2/24 hours) dropped considerably. In other words, the decrease in nectar production per leaf area was caused by leaf expansion rather than by a change in total investment in nectar. Because ants positively respond to higher nectar concentrations and nectar production per nectary (Bixenmann et al. 2011), the constant nectar production rate per nectary in this study suggests that the same number of ants will visit nectaries and patrol a leaf regardless of the leaf size. Thus, the area an ant would have to patrol to effectively reduce herbivory would become increasingly larger and dilute the effectiveness of the ant bodyguard. These results highlight the important difference between total defense investment per leaf and investment per leaf tissue (Koricheva 1999) and the effects the two can have on ecological outcomes.

Phenolics and saponins were most concentrated in the youngest leaves (~29% DW), suggesting that defense at this stage is important. Furthermore, both phenolic and saponin concentration decreased as leaves expanded. This may be because: 1) investment in phenolics and saponins happened as the young leaf was formed and were then diluted, or 2) phenolics and saponins were continuously synthesized during young leaf expansion, but, at a rate that still resulted in a decrease in concentration. Brenes-Arguedas et al (2006) found that flavanoid content (a class of phenolics) increased throughout leaf expansion in *I. goldmanii* and during the first half of leaf expansion in *I. umbellifera*. However, the flavanoid concentration during leaf expansion decreased for both species, by 44 and 65% respectively, despite the continued investment in phenolic content. In the present study the total phenolic concentration decreased by half during leaf expansion despite a tenfold increase in leaf area. This suggests that total phenolic content increased during leaf expansion but at a rate that resulted in decreasing phenolic concentration. Similarly, saponins in *I. marginata* decreased by almost half (49%) while the leaf area increased nearly nine-fold. Despite the continued synthesis of phenolics and saponins during leaf expansion, the concentration in the youngest leaves was still 50% higher than the concentrations of older leaves near the adult size. Thus, it appears young leaves are physiologically able to synthesize phenolics and saponins during leaf expansion but do not maintain the same concentration throughout development. This suggests that there is an adaptive significance to investing relatively more defense to younger, smaller leaves.

Tyrosine concentration had no relationship with young leaf size and remained at 7% of leaf dry weight throughout development. This indicates that the total tyrosine content per leaf had to increase as young leaves expanded in order to maintain the same

concentration. Indeed, Lokvam et al. (2006) also showed that total tyrosine content in *I. umbellifera* increased to maintain a constant concentration until the leaf matured, at which point tyrosine was almost completely removed. The continual investment in tyrosine during leaf expansion and its subsequent removal from mature leaves suggest that tyrosine is metabolically labile and can therefore be reallocated to other tissue. Therefore, an increasing investment in tyrosine during leaf expansion would not be a lost investment, as it is removed from mature leaves when they become effectively defended by toughness.

Conclusion

Nitrogen content is the highest in young leaves and decreases during leaf expansion (Kursar and Coley 2003). High nitrogen content makes young leaves more nutritious for herbivores, but also more valuable to the plant. In addition, the proportion of tissue lost as a young leaf, becomes a larger proportion of the mature leaf as it expands (Hunt et al. 1995). Therefore the youngest leaves should invest relatively more in defense. In this report we demonstrate that indeed plants do invest relatively more in nectary tissue, extrafloral nectar production, and defensive chemistry (except for tyrosine) in the youngest leaves. Furthermore, the early investment in all defenses and the lack of any negative correlations among defenses suggest that there is not a trade-off among defenses but rather, an additive effect resulting in better protection for the youngest leaves. Furthermore, the decrease in phenolic concentration (50%) during normal young leaf expansion in *Inga* is over twice the amount that young leaves can induce these defenses (23%; Bixenmann In Prep.) Thus, protecting younger, smaller

leaves appears to be more critical than protecting the same leaves in the future. While the optimal defense hypothesis predicts trade-offs between investments, it also predicts that more valuable tissue will be defended more thoroughly (McCall and Fordyce 2010). Therefore, our results suggest that young leaves of tropical trees, especially the youngest leaves, have a higher potential value than mature leaves and therefore require much more protection.

Table 4.1 ANCOVA table for the response variables. Models started with all factors and were reduced using "step" in R.

Source	Df	Sum Sq	Mean Sq	F-value	P-value
Volume					
Plant species	2	261.24	130.619	5.979	P<0.010
Residuals	67	1463.69	21.846		
log(Average Nectary Area)					
Plant species x Gap	5	6.7115	1.3423	6.262	P<0.001
Residuals	74	15.8633	0.21437		
log(Nectary area/Leaf area)					
Leaf expansion	1	176.785	176.785	150.414	P<0.001
Plant species	2	19.928	9.964	8.478	P<0.001
Residuals	76	89.324	1.175		
Nectar production (µg sugar/nectary/24 hr)					
Plant species	2	639559	319780	2.271	0.111
Residuals	69	9714596	140791		
log(Nectar prodcution (µg sugar/cm^2/24 hr))					
Leaf expansion	1	111.67	111.671	34.432	P<0.001
Residuals	70	227.03	3.243		
Phenolics (mg/mg)					
Leaf expansion	1	0.14351	0.143513	17.379	P<0.001
Plant species	2	0.07976	0.039880	4.829	P<0.050
Residuals	50	0.4129	0.008258		
Saponins (mg/mg)					
Leaf expansion	1	0.017516	0.0175160	43.861	P<0.001
Percent damage	1	0.0044131	0.0044131	11.051	P<0.010
Gap	1	0.0065342	0.0065342	16.362	P<0.010
Residuals	11	0.0043929	0.0003994		
Tyrosine (mg/mg)					
Leaf expansion	2	0.000102	0.000102	0.011	P<0.918
Residuals	23	0.214935	0.009345		

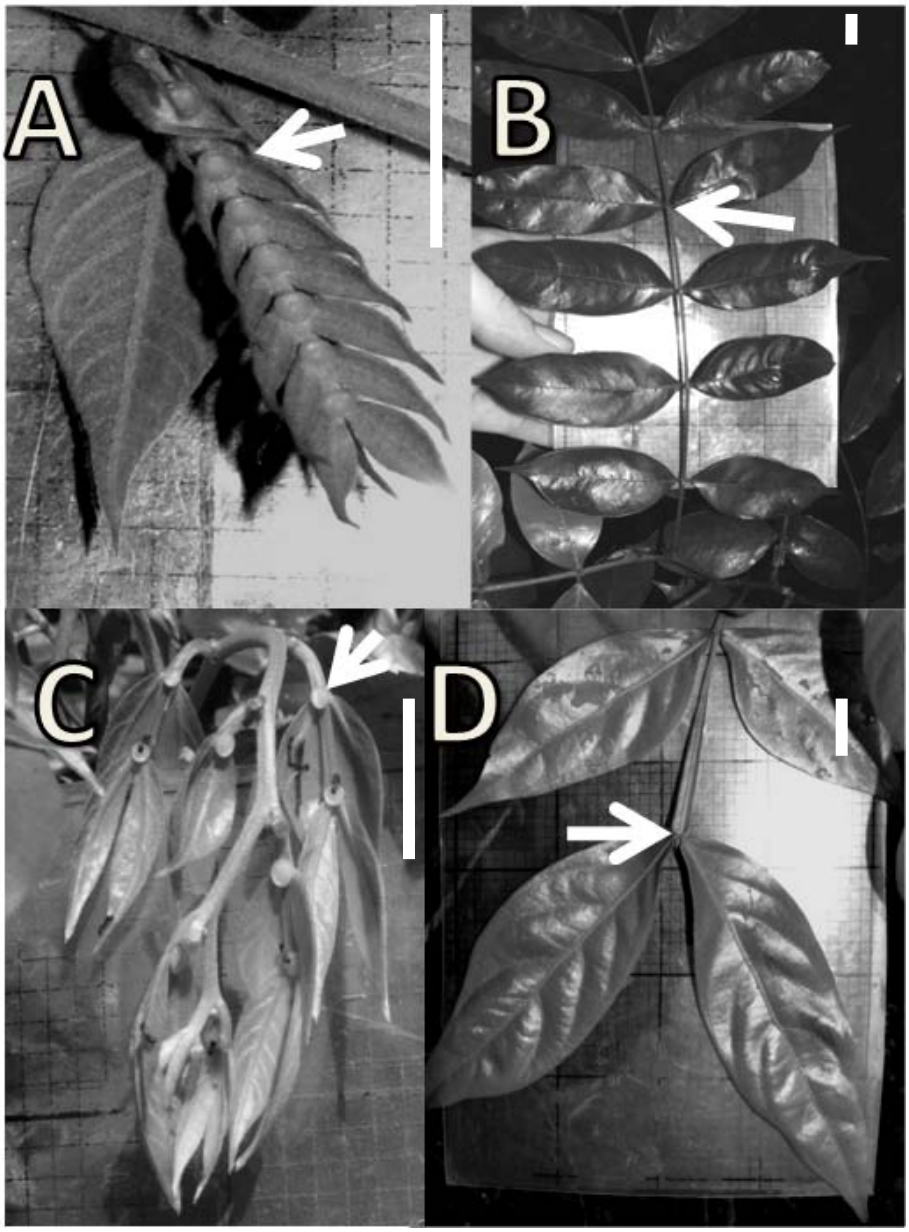

Figure 4.1 The ratio of extrafloral nectary to leaf area significantly decreases as young leaves expand. Nectary area does not change during leaf expansion, but leaf area does. White arrows point to extrafloral nectaries on leaves less than 25% (left) and greater than 80% (right) of adult size on *I. multijuga* (A and B) and *I. marginata* (C and D). Scale bars in the top right corner of each picture represent 1.27 cm.

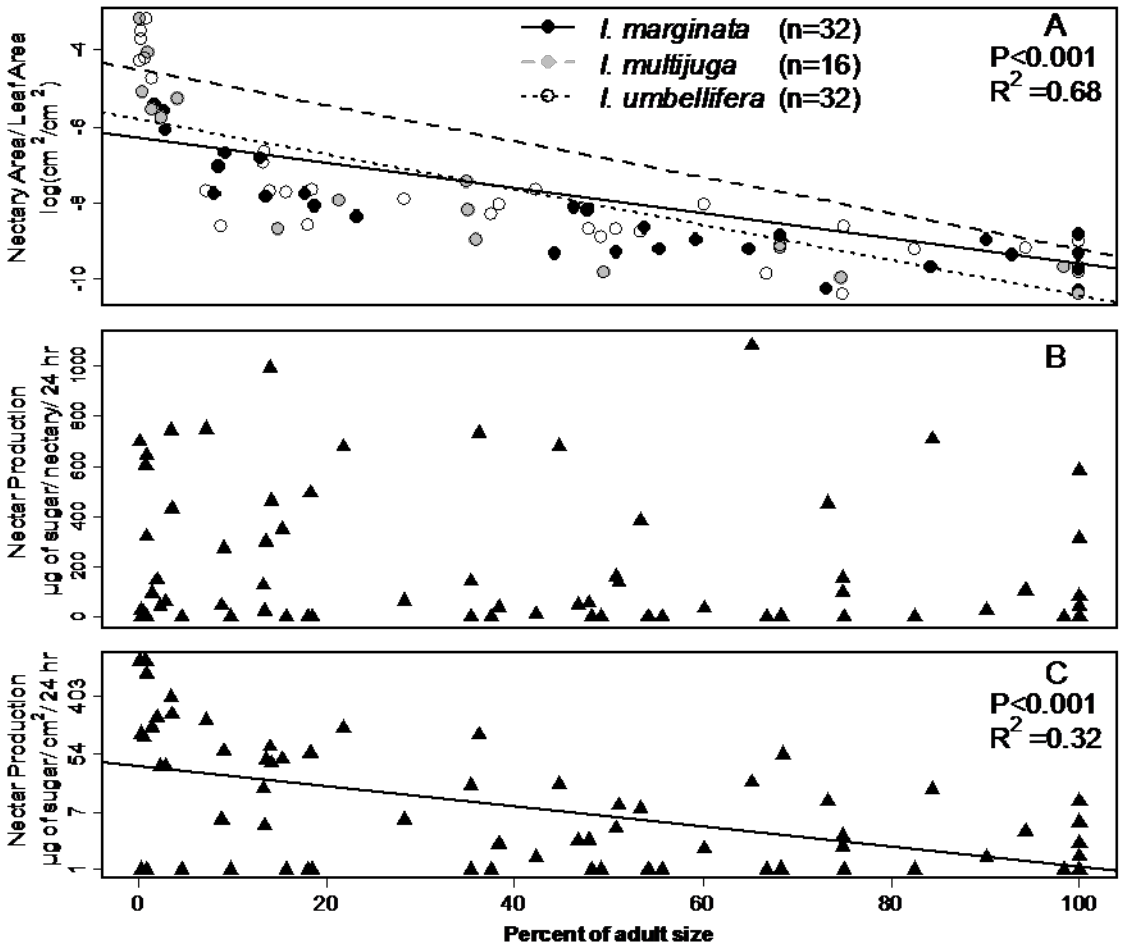

Figure 4.2 Nectary area and nectar production per leaf area decrease as leaves expand. The ratio of nectary area to leaf area for *I. marginata* (filled circles and solid line), *I. multijuga* (grey circles and dashed line), and *I. umbellifera* (open circles and dotted line) significantly decreased as leaves expanded (A; $F_{1, 76}$=150.41, R^2=0.68, P<0.001) and the mean nectary area/ leaf area was significantly different among species ($F_{2, 76}$=8.48, P<0.001). There was no change in nectar production per nectary for any species (B, NS), but nectar production per leaf area significantly decreased for all species (C; $F_{1, 70}$=34.43, R^2=0.32, P<0.001). Because nectar production for all three *Inga* species responded similarly, they are pooled in panels (B) and (C) as filled triangles.

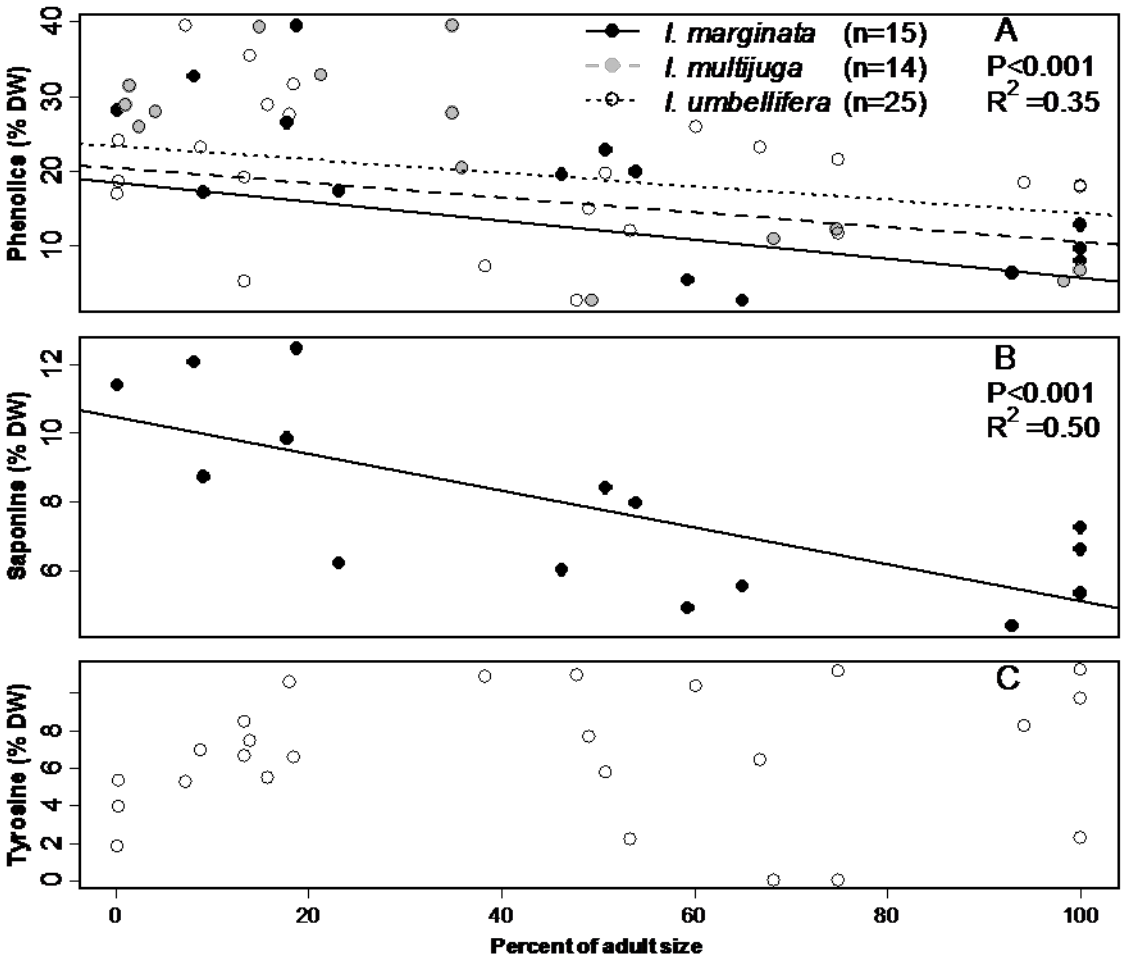

Figure 4.3 Phenolics and saponins decrease as young leaves expand. Phenolic concentration for *I. marginata* (filled circles and solid line), *I. multijuga* (filled triangles and dashed line), and *I. umbellifera* (open circles and dotted line) significantly decreased as leaves expanded (A; $F_{1, 50}$=17.38, R^2=0.35, P<0.001) and phenolic concentration was significantly different among species ($F_{2, 50}$=4.83, P<0.001). Saponins were only present in *I. marginata* and significantly decreased as leaves expanded (B; $F_{1, 11}$=43.86, P<0.001, R^2=0.50). There was no relationship between tyrosine and leaf expansion in *I. umbellifera* (C; NS).

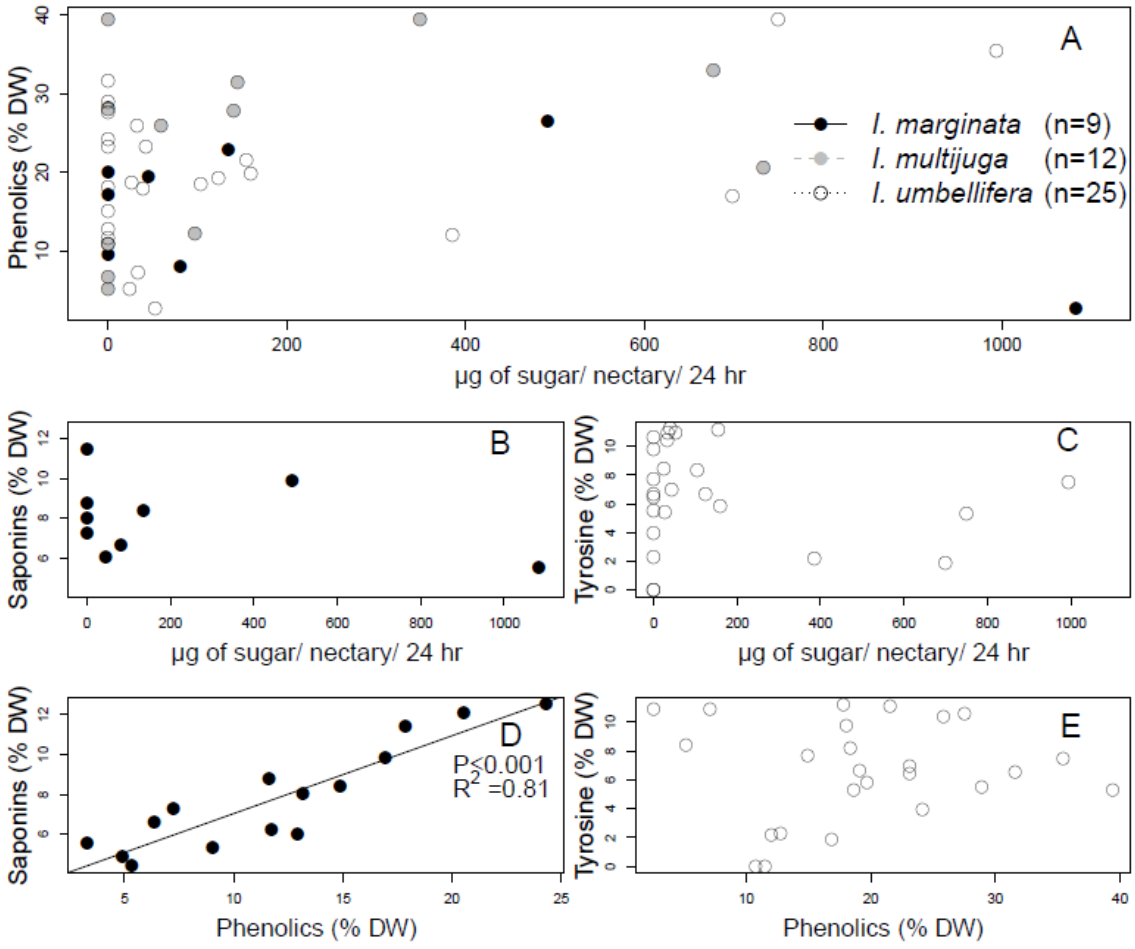

Figure 4.4 Correlations among response variables. There was no significant relationship between nectar production and phenolics (A), saponins (B), or tyrosine (C). However, there was a significant correlation between saponins and phenolics (D; $P<0.001$, $R^2=0.81$). But, there was no significant relationship between phenolics and tyrosine (E). Responses are for *I. marginata* (filled circles and solid line), *I. multijuga* (grey circles), *and I. umbellifera* (open circles).

References

Agrell, J., W. Oleszek, A. Stochmal, M. Olsen, and P. Anderson. 2003. Herbivore-induced responses in alfalfa (*Medicago sativa*). Journal of Chemical Ecology **29**:303-320.

Bixenmann, R. J., P. D. Coley, and T. A. Kursar. 2011. Is extrafloral nectar production induced by herbivores or ants in a tropical facultative ant-plant mutualism? Oecologia **165**:417-425.

Boege, K. 2004. Induced responses in three tropical dry forest plant species - direct and indirect effects on herbivory. Oikos **107**:541-548.

Brenes-Arguedas, T., M. W. Horton, P. D. Coley, J. Lokvam, R. A. Waddell, B. E. Meizoso-O'Meara, and T. A. Kursar. 2006. Contrasting mechanisms of secondary metabolite accumulation during leaf development in two tropical tree species with different leaf expansion strategies. Oecologia **149**:91-100.

Brunt, C., J. Read, and G. D. Sanson. 2006. Changes in resource concentration and defence during leaf development in a tough-leaved (*Nothofagus moorei*) and soft-leaved (*Toona ciliata*) species. Oecologia **148**:583-592.

Coley, P. D. and T. M. Aide. 1991. Comparisons of herbivory and plant defenses in temperate and tropical broad-leaved forests. Pages 25-49 *in* P. W. Price, T. M. Lewinsohn, G. W. Fernandes, and W. W. Benson, editors. Plant-animal interactions: Evolutionary ecology in tropical and temperate regions. John Wiley & Sons, Inc., New York.

Coley, P. D. and J. A. Barone. 1996. Herbivory and plant defenses in tropical forests. Annual Review of Ecology and Systematics **27**:305-335.

Coley, P. D., J. Lokvam, K. Rudolph, K. Bromberg, T. E. Sackett, L. Wright, T. Brenes-Arguedas, D. Dvorett, S. Ring, A. Clark, C. Baptiste, R. T. Pennington, and T. A. Kursar. 2005. Divergent defensive strategies of young leaves in two species of *Inga*. Ecology **86**:2633-2643.

Croat, T. B. 1978. Flora of Barro Colorado Island. Stanford University Press, Stanford.

Elias, T. S. 1983. Extrafloral nectaries: Their structure and distribution. Pages 174-203 *in* B. Bentley and T. S. Elias, editors. The Biology of Nectaries. Columbia University Press, New York.

Heil, M., B. Fiala, B. Baumann, and K. E. Linsenmair. 2000. Temporal, spatial and biotic variations in extrafloral nectar secretion by *Macaranga tanarius*. Functional Ecology **14**:749-757.

Holdridge, L., W. Grenke, W. Hatheway, T. Liang, and J. Tosi. 1971. Forest environments in tropical life zones: A pilot study. Pergamon Press, Oxford.

Hunt, T. E., L. G. Higley, and J. F. Witkowski. 1995. Bean Leaf Beetle injury to seedling Soybean: Consumption, effects of leaf expansion, and economic injury levels. Agronomy Journal **87**:183-188.

Koptur, S. 1984. Experimental evidence for defense of *Inga* (Mimosoideae) saplings by ants. Ecology **65**:1787-1793.

Koptur, S. 1985. Alternative defenses against herbivores in *Inga* (Fabaceae: Mimosoideae) over an elevational gradient. Ecology **66**:1639-1650.

Koricheva, J. 1999. Interpreting phenotypic variation in plant allelochemistry: problems with the use of concentrations. Oecologia **119**:467-473.

Kost, C. and M. Heil. 2008. The defensive role of volatile emission and extrafloral nectar secretion fro lima bean in nature. Journal of Chemical Ecology **34**:2-13.

Kursar, T. A. and P. D. Coley. 2003. Convergence in defense syndromes of young leaves in tropical rainforests. Biochemical Systematics and Ecology **31**:929-949.

Leigh, E. G. 1999. Tropical forest ecology: A view from Barro Colorado Island. Oxford University Press, Oxford.

Lokvam, J., T. Brenes-Arguedas, J. S. Lee, P. D. Coley, and T. A. Kursar. 2006. Allelochemic function for a primary metabolite: the case of L-tyrosine hyper-production in *Inga umbellifera* (Fabaceae). American Journal of Botany **93**:1109-1115.

Lokvam, J., P. D. Coley, and T. A. Kursar. 2004. Cinnamoyl glucosides of catechin and dimeric procyanidins from young leaves of *Inga umbellifera* (Fabaceae). Phytochemistry **65**:351-358.

Lokvam, J. and T. A. Kursar. 2005. Divergence in structure and activity of phenolic defenses in young leaves of two co-occurring *Inga* species. Journal of Chemical Ecology **31**:2563-2580.

Massey, F. P., M. C. Press, and S. E. Hartley. 2005. Long- and short-term induction of defences in seedlings of *Shores leprosula* (Dipterocarpaceae): Support for the carbon:nutrient balance hypothesis. Journal of Tropical Ecology **21**:195-201.

McCall, A. C. and J. A. Fordyce. 2010. Can optimal defence theory be used to predict the distribution of plant chemical defences? Journal of Ecology **98**:985-992.

Mondor, E. B. and J. F. Addicott. 2003. Conspicuous extra-floral nectaries are inducible in *Vicia faba*. Ecology Letters **6**:495-497.

Pacini, E. and M. Nepi. 2007. Nectar production and presentation. Pages 167-214 *in* S. W. Nicolson, M. Nepi, and E. Pacini, editors. Nectaries and Nectar. Springer, Dordrecht, The Netherlands.

Pennington, T. D. 1997. The Genus Inga. Royal Botanic Gardens, Kew.

Potter, D. A. and T. W. Kimmerer. 1989. Inhibition of herbivory on young holly leaves: Evidence for the defensive role of saponins. Oecologia **78**:322-329.

R Development Core Team. 2009. R: A language and environment for statistical computing. R Foundation for Statistical Computing Vienna, Austria.

Rhoades, D. F. 1979. Evolution of plant chemical defense against herbivores. . Pages 1-55 *in* G. A. Rosenthal and D. H. Janzen, editors. Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, New York:.

Richards, L. A. and D. M. Windsor. 2007. Seasonal variation of arthropod abundance in gaps and the understorey of a lowland moist forest in Panama. Journal of Tropical Ecology **23**:169-176.

Yadav, J., C.-W. Tan, and S.-Y. Hwang. 2010. Spatial variation in foliar chemicals within radish (*Raphanus sativus*) plants and their effects on performance of *Spodoptera litura*. Environmental Entomology **39**:1990-1996.